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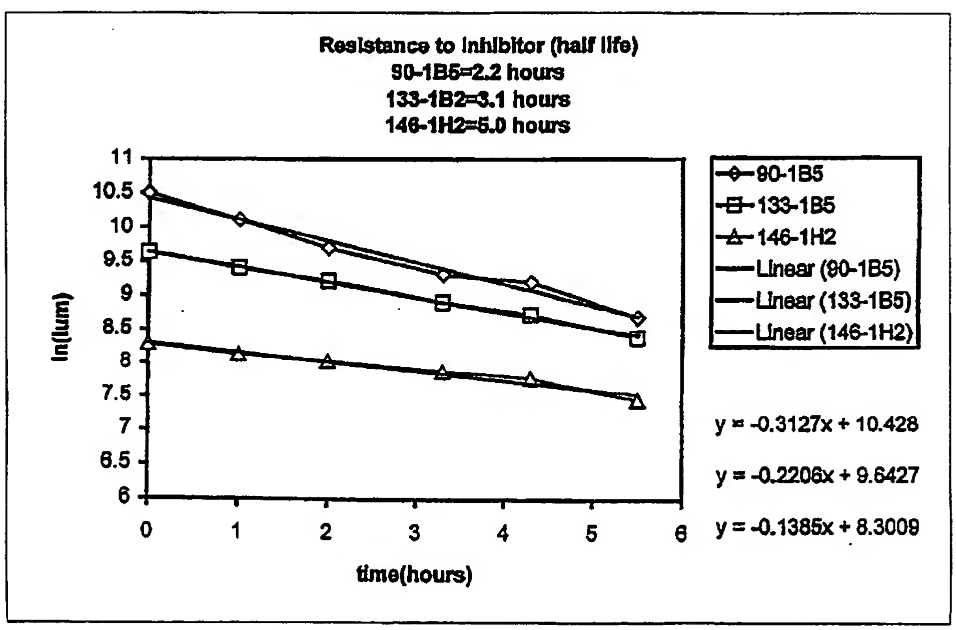
(54) Title: THERMOSTABLE LUCIFERASES FROM PHOTURIS PENNSYLVANICA AND PYROPHORUS PLAGIOPHTHALAMUS AND METHODS OF PRODUCTION



(57) Abstract: Luciferase enzymes with greatly increased thermostability, e.g., at least half lives of 2 hours at 50 °C, cDNAs encoding the novel luciferases, and hosts transformed to express the luciferases, are disclosed. Methods of producing the luciferases include recursive mutagenesis. The luciferases are used in conventional methods, some employing kits.

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THERMOSTABLE LUCIFERASES FROM PHOTURIS PENNSYLVANICA AND PYROPHORUS PLAGIOPHTHALAMUS AND METHODS OF PRODUCTION

Statement of Government Rights

The invention was made with grants from the Government of the United States of America (grants 1R43 GM506 23-01 and 2R44 GM506 23-02 from the National Institutes of Health and grants ISI-9160613 and III-9301865 from the National Science Foundation). The Government may have certain rights to the invention.

Field of the Invention

The invention is directed to mutant luciferase enzymes having greatly increased thermostability compared to natural luciferases or to luciferases from which they are derived as measured, e.g., by half-lives of at least 2 hours at 50°C in aqueous solution. The invention includes mutant luciferase enzymes that are resistant to inhibition by a substrate inhibitor, e.g., a substrate analog. The invention is also drawn to polynucleotides encoding the novel luciferases, and to hosts transformed to express the luciferases. The invention is further drawn to methods of producing luciferases with increased thermostability and the use of these luciferases in any method in which previously known luciferases are conventionally employed. Some of the uses employ kits. The invention also provides a method of producing a polynucleotide sequence encoding an enzyme that is resistant to inhibition by an inhibitor, and a method which yields a polynucleotide sequence encoding an enzyme having enhanced enzymological properties.

Background of the Invention

Luciferases are defined by their ability to produce luminescence. Beetle luciferases form a distinct class with unique evolutionary origins and chemical mechanisms (Wood, 1995).

Although the enzymes known as beetle luciferases are widely recognized for their use in highly sensitive luminescent assays, their general utility has been limited due to low thermostability. Beetle luciferases having amino acid sequences encoded by cDNA sequences cloned from luminous beetles are not stable even at moderate temperatures. For example, even the most stable of the

luciferases, Luc*Ppe2*, obtained from a firefly has very little stability at the moderate temperature of 37°C. Firefly luciferases are a sub-group of the beetle luciferases. Historically, the term "firefly luciferase" referred to the enzyme Luc*Ppy* from a single species *Photinus pyralis* (Luc + is a mutant version of LucPpy, see U.S. Patent No. 5,670,356).

Attempts have been reported to mutate natural cDNA sequences encoding luciferase and to select mutants for improved thermostability (White et al., 1994; from *P. pyralis,* and Kajiyama and Nekano, 1993; from *Luciola lateralis*.) However, there is still a need to improve the characteristics and versatility of this important class of enzymes.

Summary of the Invention

The invention is drawn to novel and remarkably thermostable luciferases, including luciferase enzymes with half-lives of at least 2 hours at 50°C, or at least 5 hours at 50°C, in an aqueous solution. As described hereinbelow, after 2 hours at 50°C in an aqueous solution, a thermostable luciferase of the invention lost less than 5% luminescence activity. The mutant luciferases of the present invention display remarkable and heretofore unrealized thermostability at 22°C in an aqueous solution and at temperatures at least as high as 60°C in an aqueous solution. For example, the luciferases of the invention are thermostable for at least 10 hours at 50°C; for at least 2 hours, preferably at least 5 hours, more preferably at least 10 hours, and even more preferably at least 24 hours, at 60°C; and/or for at least 100 days, preferably at least 200 days, more preferably at least 500 days, and even more preferably at least 800 days, at 22°C, in aqueous solution. For example, after 30 days at 22°C in an aqueous solution, a thermostable luciferase of the invention lost less than 5% luminescence activity. Preferably, the thermostable luciferases of the invention have enhanced luminescence intensity, enhanced signal stability, enhanced substrate utilization, and/or decreased Km, relative to a reference, e.g., a native wild-type, luciferase. The invention is further directed to the mutant luciferase genes (e.g., cDNA or RNA) which encode the novel luciferase enzymes. The terminology used herein is, e.g., for the mutants isolated in experiment 90, plate number 1, well B5, the *E. coli* strain is 90-1B5, the mutant gene is *luc90-1B5*, and the mutated luciferase is Luc90-1B5.

As defined herein, a "thermostable" enzyme, e.g., a luciferase, or an enzyme which has "thermostability", is an enzyme which under certain conditions, e.g., at certain temperature, in aqueous solution and/or for certain periods of time, has an increased retention of activity relative to a reference enzyme. For example, for a thermostable luciferase, a reference luciferase may be native wild-type luciferase or recombinant wild-type luciferase. Preferably, for beetle luciferases, the activity is luminescence under conditions of saturation with luciferin and ATP. One measure of thermostability of an enzyme is the half-life of the enzyme in an aqueous solution (the time over which 50% of the activity is lost) at a stated temperature.

The invention further encompasses expression vectors and other genetic constructs containing the mutant luciferases, as well as hosts, bacterial and otherwise, transformed to express the mutant luciferases. The invention is also drawn to compositions and kits which contain the novel luciferases, and use of these luciferases in any methodology where luciferases are employed.

Various means of random mutagenesis were applied to a luciferase gene (nucleotide sequence), most particularly gene synthesis using an error-prone polymerase, to create libraries of modified luciferase genes. This library was expressed in colonies of *E. coli* and visually screened for efficient luminescence to select a subset library of modified luciferases. Lysates of these *E. coli* strains were then made, and quantitatively measured for luciferase activity and thermostability. From this, a smaller subset of modified luciferases was chosen, and the selected mutations were combined to make composite modified luciferases. New libraries were made from the composite modified luciferases by random mutagenesis and the process was repeated. The luciferases with the best overall performance were selected after several cycles of this process.

Methods of producing improved luciferases include directed evolution using a polynucleotide sequence encoding a first beetle luciferase as a starting (parent) sequence, to produce a polynucleotide sequence encoding a second luciferase with increased thermostability, compared to the first luciferase, while maintaining other characteristics of the enzymes. A cDNA designated *lucPpe2* encodes a firefly luciferase derived from *Photuris pennsylvanica* that displays increased thermostability as compared to the widely utilized luciferase

designated Luc*Ppy* from *Photinus pyralis*. The cDNA encoding Luc*Ppe2* was isolated, sequenced and cloned (see Leach et al., 1997). A mutant of this gene encodes a first luciferase Luc*Ppe2* [T249M]. However, the methods of the invention are not limited to use with a polynucleotide sequence encoding a beetle luciferase, i.e., the methods of the invention may be employed with a polynucleotide sequence encoding other enzymes.

In an embodiment of a mutant luciferase, the amino acid sequence is that of Luc*Ppe2* shown in Figure 45 with the exception that at residue 249 there is a M (designated T249M) rather than the T reported by Leach et al. The underlined residue (249) shows mutation from T to M. This enzyme produced approximately 5-fold more light *in vivo* when expressed in *E. coli.*

Diluted extracts of recombinant *E. coli* that expressed mutant luciferases made by the methods of the invention were simultaneously screened for a plurality of characteristics including light intensity, signal stability, substrate utilization (K_m), and thermostability. A fully automated robotic system was used to screen large numbers of mutants in each generation of the evolution. After several cycles of mutagenesis and screening, thereby creating mutant libraries of luciferases, an increased thermostability compared to Luc*Ppe2* [T249M] of about 35°C was achieved for clone Luc90-1B5 which also essentially maintained enzymatic activity (there was only negligible loss in activity of 5%) when kept in aqueous solution over 2 hours at 50°C, 5 hours at 65°C, or over 6 weeks at 22°C.

Mutant luciferases of the present invention display increased thermostability for at least 2 hours at 50°C, preferably at least 5 hours at 50°C, and in the range of at least 2 hours, preferably at least 24 hours, and more preferably at least 50 hours, at temperatures including 50°C, 60°C, and/or at temperatures up to 65°C. In particular, the present invention comprises thermostable mutant luciferases which, when solubilized in a suitable aqueous solution, have a thermostability greater than about 2 hours at about 50°C, more preferably greater than about 10 hours at 50°C, and more preferably still greater than 5 hours at 50°C. The present invention also comprises mutant luciferases which, when solubilized in a suitable aqueous solution, have a thermostability greater than about 2 hours, more preferably at least 5 hours, even more preferably greater than about 10 hours, and even more preferably still greater

than about 24 hours, at about 60°C. The present invention further comprises mutant luciferases which when solubilized in a suitable aqueous solution have a thermostability greater than about 3 months at about 22°C, and more preferably a thermostability of at least 6 months at 22°C. An embodiment of the invention is a luciferase mutant having thermostability at 65°C, wherein a loss of activity of about 5-6% was found after 6 hours (equivalent to a half-life of 2 days). The half-lives of enzymes from the most stable clones of the present invention, extrapolated from data showing small relative changes, is greater than 2 days at 65°C (corresponding to 6% loss over 6 hours), and about 2 years at 22°C (corresponding to 5% loss over 9 weeks).

In particular, the invention comprises luciferase enzymes with embodiments of amino acid sequences disclosed herein (e.g., mutant luciferases designated Luc49-7C6, Luc78-0B10; Luc90-1B5, Luc133-1B2, and Luc146-1H2, as well as all other beetle luciferases that have thermostability as measured in half-lives of at least 2 hours at 50°C. The invention also comprises mutated polynucleotide sequences encoding luciferase enzymes containing any single mutation or any combination of mutations of the type which convert an amino acid of the reference beetle luciferase into a consensus amino acid. Conserved amino acids are defined as those that occur at a particular position in all sequences in a given set of related enzymes. Consensus amino acids are defined as those that occur at a particular position in more than 50% of the sequences in a given set of enzymes. An example is the set of beetle luciferase sequences shown in Figure 19, excluding Luc*Ppe2*.

Nucleotide sequences encoding beetle luciferases are aligned in Figure 19. Eleven sequences found in nature in various genera, and species within genera, are aligned, including *lucPpe2*. There are at least three mutations present in each mutant luciferase that show increased thermostability. In general, mutations are not of a conserved amino acid residue. The mutations in the mutant luciferases are indicated in Figures 22-47 by underlining.

The invention also provides methods to prepare enzymes having one or more desired properties, e.g., resistance to inhibition by a substrate analog of the enzyme or enhanced enzymological properties. The method comprises selecting at least one isolated polynucleotide sequence encoding an enzyme with the

desired property, e.g., an enzymological property, from a first population of mutated polynucleotide sequences. The selected, isolated polynucleotide sequence is then mutated to yield a second population of mutated polynucleotide sequences. Preferably, a mixture of selected isolated polynucleotide sequences are mutated to yield a second population of mutated polynucleotide sequences. The process may be repeated until a further polynucleotide sequence is obtained, e.g., selected and/or isolated, which further polynucleotide sequence encodes an enzyme which has at least one of the desired properties. As used herein, the terms "isolated and/or "purified" refer to *in vitro* isolation of a RNA, DNA or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide, e.g., so that it can be sequenced, replicated, and/or expressed.

Brief Description of the Drawings

Figure 1 is a graphical representation of thermostability at 37°C of Luc*Ppe2*[T249M]; Luc39-5B10; and Luc49-7C6, normalized to t = 0 [the X-axis is time in minutes; the Y-axis is % remaining activity; and "t" is time].

Figure 2 is a graphical representation of the remaining activity of Luc49-7C6 and Luc78-0B10 at 50°C normalized to a t = 0 reading [the X-axis is time in hours; the Y-axis is % remaining activity; and t is time].

Figure 3 is a graphical representation of the luminescence produced by Luc49-7C6 and Luc78-0B10 at 60°C normalized to t = 0 [the X-axis is time in hours; the Y-axis is % remaining activity; and t is time].

Figure 4 is a graphical representation of thermostability of luciferases, Luc*Ppe2*[T249M]; Luc49-7C6; and Luc78-0B10 thermostability at 22°C [the X-axis is time in days; the Y-axis is normalized light units].

Figure 5 is a graphical representation of the observed log luminescence produced by (Y) Luc78-0B10 compared to log luminescence predicted by the regression equation $Y = 0.0043X + 10.91$; the half life of the enzyme is calculated as 144 hours (6 days) [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 6 is a graphical representation of the observed log luminescence produced by Luc78-0B10 at 60°C compared to the log luminescence calculated by the regression equation $Y = 0.154X + 10.86$; the half life of the enzyme is

calculated as 38 hours (1.58 days) [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 7 is a graphical representation of the observed log luminescence produced by Luc49-7C6 at 50°C compared to log luminescence predicted by the regression equation $Y = -0.0059X + 8.757$; the half-life of the enzyme is calculated as 100.5 hours (4.2 days) [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 8 is a graphical representation of the observed log luminescence produced by Luc49-7C6 at 60°C compared to the log luminescence calculated by the regression equation $Y = -0.169X + 8.647$; the calculated half-life of the enzymes is 2.9 hours (the X-axis is time in hours; the Y-axis is log luminescence).

Figure 9 is a graphical representation of the observed log luminescence produced by Luc78-0B10 at 22°C compared to a predicted log luminescence, the half-life of the enzyme is 109 days [the X-axis is time in days; the Y-axis is log luminescence].

Figure 10 is a graphical representation of the observed luciferase log luminescence produced by Luc49-7C6 at 22°C compared to a predicted log luminescence; the half-life of the enzyme is 64 days [the X-axis is time in days; the Y-axis is log luminescence].

Figure 11 is a graphical representation of the observed log luminescence produced by luciferase Luc49-7C6 at 37°C compared to predicted log luminescence [the X-axis is time in minutes; the Y-axis is log luminescence].

Figure 12 is a graphical representation of the observed log luminescence produced by luciferase Luc*Ppe2* [T249M] at 22°C compared to predicted log luminescence [the X-axis is time in days; the Y-axis is log luminescence].

Figure 13 is a graphical representation of the observed log luminescence produced by luciferase Luc*Ppe2* [T249M] at 37°C compared to predicted log luminescence [X-axis is time in minutes; Y-axis is log luminescence].

Figure 14 is a flow chart showing steps for an assay of *in vivo* and *in vitro* luciferase luminescence (Li); enzyme stability (τ); assay kinetics (S); and substrate binding (Km).

Figure 15 is a schematic representation of a table top layout robot.

Figure 16A is a graphical representation of luciferase mutant Luc90-1B5 luminescence measured at 65°C, pH 6.5 (the X-axis is time in hours; the Y-axis is % luminescence).

Figure 16B is a graphical representation of luciferase mutant Luc90-1B5 luminescence at 22°C, pH 6.5 (the X-axis is time in days; the Y-axis is % luminescence).

Figure 17 is a diagram showing the evolutionary relationships among beetle luciferases based on amino acid sequences.

Figure 18A is a representation of the secondary structures of beetle luciferase enzymes (helices are symbolized by cylinders, sheets by collections of arrows, loops connect helices with sheets).

Figure 18B shows the amino acids (tertiary structures) of the Luc*Ppe2* luciferase, wherein small spirals correspond to cylinders of Figure 18A.

Figure 18C shows that the general beetle architecture matches (is superimposed on) that of Luc90-1B5.

Figure 19A presents alignment of the amino acid sequence (SEQ ID Nos:27-37) for luciferases from various beetle species (Lcr, Lla, Lmi, Pmi, Ppy, Lno, Ppe1, Phg, GR, YG, Ppe2, respectively) and luciferases of the present invention (Luc49-7C6; Luc78-0B10; Luc90-1B5, Luc133-1B2; and Luc146-1H2, SEQ ID Nos. 14, 19, 24, 44, and 45, respectively); the sequences are aligned, spaces where sequences cannot be aligned are shown by dots (e.g.,. . .); only amino acids that differ in the luciferases of the present invention from those of some beetle species are shown, not the full sequences. "Cons" is a sequence showing conserved amino acids by single letters, and indicates non-conserved amino acids by "-" .

Figure 19B presents alignment of the amino acid sequence (SEQ ID Nos:27-37) for luciferases from various beetle species (Lcr, Lla, Lmi, Pmi, Ppy, Lno, Ppe1, Phg, GR, YG, Ppe2) and luciferases of the present invention (Luc30-4B02 and Luc81-6G01, SEQ ID Nos. 47 and 26, respectively); the sequences are aligned, spaces where sequences cannot be aligned are shown by dots (e.g.,. . .); amino acids that differ in the luciferases of the present invention from those of some beetle species are shown in bold.

Figure 19C presents alignment of the amino acid sequence (SEQ ID NOs:27-34 and 36-37) for luciferases from various beetle species (Lcr, Lla, Lmi, Pmi, Ppy, Lno, Ppe1, Phg, YG, Ppe2, Ppl); the sequences are aligned, spaces where sequences cannot be aligned are shown by dots (e.g.,. . .); in the line beneath YG, X indicates positions in YG where mutations could yield a consensus amino acid; O indicates positions in YG where mutations could not yield a consensus amino acid.

Figure 20 is the 7216 bp *Ppe2* vector map in a pRAM backbone.

Figure 21 is a bar graph comparing luminescence as expressed in recombinant colonies of *E. coli*; the colonies differ in the identity of the luciferase encoding vector (Luc+; Luc90-1B5; Luc78-1B10; Luc49-7C6; Luc*Ppe2* [T249M] and Luc*Ppe2*); in the recombinant colony shown in the Y-axis [the X-axis is normalized light units].

Figure 22 is a nucleotide (DNA) sequence (SEQ ID NO:1) encoding mutant luciferase enzyme Luc49-7C6; mutations are indicated by underlining.

Figure 23 is a nucleotide (DNA) sequence (SEQ ID NO:2) encoding mutant luciferase enzyme Luc49-6C10, mutations are indicated by underlining.

Figure 24 is a nucleotide (DNA) sequence (SEQ ID NO:3) encoding a mutant luciferase enzyme Luc49-0G12; mutations are indicated by underlining.

Figure 25 is a nucleotide (DNA) sequence (SEQ ID NO:4) encoding a mutant luciferase enzyme Luc49-7A5; mutations are indicated by underlining.

Figure 26 is a nucleotide (DNA) sequence (SEQ ID NO:5) encoding a mutant luciferase enzyme Luc49-4G11; mutations are indicated by underlining.

Figure 27 is an amino acid sequence (SEQ ID NO:14) of the mutant luciferase designated Luc49-7C6; mutations are indicated by underlining.

Figure 28 is an amino acid sequence (SEQ ID NO:15) of mutant luciferase enzyme Luc49-6C10; mutations are indicated by underlining.

Figure 29 is an amino acid sequence (SEQ ID NO:16) of mutant luciferase enzyme Luc49-0G12; mutations are indicated by underlining.

Figure 30 is an amino acid sequence (SEQ ID NO:17) of mutant luciferase enzyme Luc49-7A5; mutations are indicated by underlining.

Figure 31 is an amino acid sequence (SEQ ID NO:18) of mutant luciferase enzyme Luc49-4G11; mutations are indicated by underlining.

Figure 32 is a nucleotide (DNA) sequence (SEQ ID NO:6) encoding mutant luciferase enzyme Luc78-0B10; mutations are indicated by underlining.

Figure 33 is a nucleotide (DNA) sequence (SEQ ID NO:7) encoding mutant luciferase enzyme Luc78-0G8; mutations are indicated by underlining; X's signify unknown identities of nucleotides at certain positions.

Figure 34 is a nucleotide (DNA) sequence (SEQ ID NO:8) encoding mutant luciferase enzyme Luc78-1E1; mutations are by underlining; X's signify that the identity of a nucleotide at a position is unknown.

Figure 35 is a nucleotide (DNA) sequence (SEQ ID NO:9) encoding a mutant luciferase Luc78-2B4; underlined nucleotides are mutations; X's signify unknown identities of nucleotides at certain positions.

Figure 36 is an amino acid sequence (SEQ ID NO:19) of the mutant luciferase Luc78-0B10; underlined amino acids are mutations.

Figure 37 is an amino acid sequence (SEQ ID NO:20) of the mutant luciferase enzyme Luc78-0G8; underlined amino acids are mutations; X's signify unknown amino acids at a position.

Figure 38 is an amino acid sequence (SEQ ID NO:21) for mutant luciferase enzyme Luc78-1E1; underlined amino acids are mutations; X's signify an unknown amino acid at a position.

Figure 39 is an amino acid sequence (SEQ ID NO:22) for mutant luciferase enzyme Luc78-2B4; underlined amino acids are mutations; X's signify an unknown amino acid at a position.

Figure 40 is a nucleotide (DNA) sequence (SEQ ID NO:10) for encoding a mutant luciferase enzyme Luc85-4F12; underlined nucleotides are mutations; X's signify an unknown amino acid at that position.

Figure 41 is an amino acid listing (SEQ ID NO:23) for a mutant luciferase enzyme Luc85-4F12; underlined amino acids are mutations; X's signify an unknown amino acid at that position.

Figure 42 is a nucleotide (DNA) sequence (SEQ ID NO:11) encoding mutant luciferase enzyme Luc90-1B5; underlined nucleotides are mutations.

Figure 43 is an amino acid sequence (SEQ ID NO:24) for the mutant luciferase designated Luc90-1B5; underlined amino acids are mutated positions.

Figure 44 is a nucleotide (DNA) sequence (SEQ ID NO:12) encoding luciferase enzyme Luc*Ppe2* [T249M].

Figure 45 is an amino acid sequence (SEQ ID NO:25) for Luc*Ppe2* [T249M]; the underlined amino acid is a mutation from Thr to Met at residue 249.

Figure 46 is an amino acid sequence (SEQ ID NO:26) for luciferase enzyme Luc*Pp1*81-6G1; underlined amino acids are mutations from a starting sequence; X shows ambiguity.

Figure 47 is a nucleotide (DNA) sequence (SEQ ID NO:13) encoding luciferase enzyme Luc81-6G1; underlined nucleotides are mutations.

Figure 48 is a graphical representation of mutant luciferases Luc49-7C6 and Luc78-0B10 luminescence at 60°C normalized to t = 0 [the X-axis is time in hours, the Y-axis is log normalized luminescence].

Figure 49 is a graphical representation of luciferases Luc*Ppe2* [T249M], Luc49-7C6, and Luc78-0B10, thermostability at 4°C, normalized to initial values [the X-axis is time in days; Y is log normalized light units].

Figure 50 is a graphical representation of mutant luciferases Luc49-7C6 and Luc78-0B10 luminescence at 50°C normalized to t = 0 [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 51 is a graphical representation of mutant luciferases Luc49-7C6 and Luc78-0B10 luminescence at 50°C normalized at t = 0.

Figure 52 is a graphical representation of mutant luciferases Luc49-7C6 and Luc78-0B10 luminescence at 60°C normalized to t = 0 [the X-axis is time in hours; the Y-axis is luminescence].

Figure 53 is a graphical representation of luciferases LucPpe2 [T249M], Luc49-7C6, and Luc78-0B10 thermostability at 22°C [the X-axis is time in days; the Y-axis is log luminescence].

Figure 54A is a graphical representation of luminescence of Luc90-1B5; Luc133-1B2; and Luc146-1H2, at pH 4.5 and 48°C, normalized to t = 0.

Figure 54B is a graphical representation of the half-life of Luc90-1B5; Luc133-1B2; and Luc146-1H2, at pH 4.5 and 48°C. The half-life of Luc90-1B5 under these conditions is about 3 minutes, Luc133-1B2 about 20 minutes, and Luc146-1H2 about 62 minutes.

Figure 55 is a nucleotide (DNA) sequence (SEQ ID NO:42) encoding a luciferase enzyme Luc133-1B2; mutations are indicated by underlining.

Figure 56 is a nucleotide (DNA) sequence (SEQ ID NO:43) encoding a luciferase enzyme Luc146-1H2; mutations are indicated by underlining.

Figure 57 is an amino acid sequence (SEQ ID NO:44) of mutant luciferase Luc133-1B2; mutations are indicated by underlining.

Figure 58 is an amino acid sequence (SEQ ID NO:45) of mutant luciferase Luc146-1H2; mutations are indicated by underlining.

Figure 59 is a graphical representation of the signal kinetics of clones Luc49-7C6; Luc78-0B10; Luc90-1B5; Luc133-1B2; and Luc146-1H2 at pH 7.8 at room temperature.

Figure 60 is a graphical representation of the normalized luminescence at 50°C pH 7.8 of Luc49-7C6; Luc78-0B10; Luc90-1B5; Luc133-1B2; and Luc146-1H2; from t = 0 to about 8 hours.

Figure 61 is a graphical representation of the resistance of selected luciferases to a substrate inhibitor. The data is presented as the log of the luminescence versus time for Luc90-1B5; Luc133-1B5; and Luc146-1H2.

Figure 62 is a graphical representation of the log of luminescence over time at 22°C, pH 6.5 for Luc90-1B5 and LucPpe2[T249M].

Figure 63 is a graphical representation of thermostability of selected mutant luciferases and Luc*PplYG* at room temperature in aqueous solution containing 1% Triton X-100.

Figures 64 is a graphical representations of the sustained luminescence activity (expressed as luminescence/O.D.) over time for certain luciferases.

Figure 65 is a nucleotide (DNA) sequence (SEQ ID NO:46) encoding a luciferase enzyme Luc81-0B11; mutations are indicated by underlining.

Figure 66 is an amino acid sequence of mutant luciferase Luc81-0B11; mutations are indicated by underlining.

Detailed Description of the Invention

The invention relates to enzymes, e.g., beetle luciferases, that are created by mutations made in the encoding genes, generally by recursive mutagenesis, which mutated enzymes have one or more desired properties, for example, increases thermostability, increased resistance to inhibitors, and/or enhanced enzymological properties, relative to a reference enzyme, e.g., the wild-type enzyme. The polynucleotide sequence which encodes an enzyme of the invention comprises mutations that encode a plurality of amino acid substitutions relative to the polynucleotide sequence encoding the enzyme from which the enzyme of the invention was derived. For example, the invention relates to enzymes, e.g., luciferases, that are thermostable. The increased thermostability allows storage of enzymes such as luciferases without altering its activity, and improves reproducibility and accuracy of assays using the mutated luciferases. Thus, one embodiment of the invention comprises isolated polynucleotide sequences (cDNAs) which encode mutant luciferases with increased thermostability, vectors containing the polynucleotide sequences, and hosts transformed to express the polynucleotide sequences. Table 1 shows results of about 250 clones and characteristics of the luciferases from the clones including thermostability. The invention also encompasses the use of the mutant luciferases in any application where luciferases are conventionally utilized, and kits useful for some of the applications.

Unexpectedly, beetle luciferases with the sought after improved thermostability were achieved in the present invention through a process of recursive mutagenesis and selection (sometimes referred to as "directed evolution"). A strategy of recursive mutagenesis and selection is an aspect of the present invention, in particular the use of multi-parameter automated screens. Thus, instead of screening for only a single attribute such as thermostability, simultaneous screening was done for additional characteristics of enzyme activity and efficiency. By this method, one property is less likely to "evolve" at the expense of another, resulting in increased thermostability, but decreased activity, for example.

Table 1 presents examples of parameter values (Li, Tau, K_m and S, see below) derived from experiments using different luciferases as starting (parent)

sequences. The subtitles refer to designations of the temperature at which the enzyme stability was measured and the starting luciferase, e.g., Luc39-5B10 at 51°C and so forth. All parameters in each experiment are recorded as relative values to the respective starting sequence, e.g., the parameter values for the starting sequence in any experiment equals "1." (See Example 2 herein for definitions.)

Thermostability has evolved in nature for various enzymes, as evidenced by thermostable isozymes found in thermophilic bacteria. Natural evolution works by a process of random mutagenesis (base substitutions, gene deletions, gene insertions), followed by selection of those mutants with improved characteristics. The process is recursive over time. Although the existence of thermostable enzymes in nature suggests that thermostability can be achieved through mutagenesis on an evolutionary scale, the feasibility of achieving a given level of thermostability for a particular class of enzymes by using short term laboratory methods was unpredictable. The natural process of evolution, which generally involves extremely large populations and many millions of generations and genes, by mutation and selection cannot be used to predict the capabilities of a modern laboratory to produce improved genes by directed evolution until such mutants are produced.

After such success, because the overall three-dimensional structure of all beetle luciferases are quite similar, having shown it possible for one member of this class makes it predictable that high thermostability can be achieved for other beetle luciferases by similar methods. Figure 17 shows an evolutionary relationship among beetles luciferases, all of which have a similar overall architecture. The structural class to which the beetle luciferases belong is determined by the secondary structure (e.g. helices are symbolized by cylinders, sheets by collections of arrows, loops connect helices with sheets (Figure 18A). Figure 18B shows the amino acids of the Luc*Ppe2* luciferase wherein small spirals correspond to cylinders of Figure 18A; Figure 18C shows that the general beetle architecture matches (is superimposed on) that of Luc*Ppe2*. This is support for the expectation that the methods of the present invention can be generalized to all beetles luciferases.

Enzymes belong to different structural classes based on the three-dimensional arrangement of secondary elements such as helices, sheets, and loops. Thermostability is determined by how efficiently the secondary elements are packed together into a three-dimensional structure. For each structural class, there also exists a theoretical limit for thermostability. All beetle luciferases belong to a common structural class as evident by their common ancestry (Figure 17), homologous amino acid sequences, and common catalytic mechanisms.

The application of a limited number of amino acid substitutions by mutagenesis is unlikely to significantly affect the overall three-dimensional architecture (i.e., the structural class for mutant luciferases is not expected to change.) Because the theoretical limit for thermostability for any structural class is not known, the potential thermostability of beetle luciferases was not known until demonstrations of the present invention.

A priori difficulties in achieving the goals of the present invention included: 1. The types of mutations which can be made by laboratory methods are limited.

i) By random point mutation (e.g. by error-prone PCR), more than one base change per codon is rare. Thus, most potential amino acid changes are rare.

ii) Other types of random genetic changes are difficult to achieve for areas greater than 100 bp (e.g., random gene deletions or insertions).

2. The number of possible luciferase mutants that can be screened is limited.

i) Based on sequence comparisons of natural luciferases, ignoring deletions and insertions, more than 10^{189} functional enzyme sequences may be possible.

ii) If 100,000 clones could be screened per day, it would require more than 10^{179} centuries to screen all possible mutants assuming same mutant was never screened twice (actual screening rate for the present invention was less than 5000 per day).

3. The probability of finding functional improvement requiring cooperative mutations is rare (the probability of finding a specific cooperative pair is 1 out of 10^8 clones).

Thus, even if the theoretical limits of thermostability were known, because only a very small number of the possible luciferase mutants can be screened, the *a priori* probability of finding such a thermostable enzyme was low.

However, the present invention now shows that it is possible and feasible to create novel beetle luciferases having high thermostability.

a) The approximately 250 mutants produced by methods of the present invention wherein the initial sequence was from *lucPpe2* or *lucPplYG* demonstrate that it is possible and feasible for at least one member of this enzyme class to achieve high thermostability.

b) Any beetle luciferase can be improved by similar means because the luciferases belong to the same structural class.

   i) Because all beetle luciferases belong to the same structural class, they also share in the same pool of potentially stabilizing mutations (this conclusion is supported by observation that a high percentage of the stabilizing mutations found in the clones of the present invention were conversions to "consensus amino acids" in other beetle luciferases that is, amino acids that appear in the majority of beetle luciferase sequences (see Figure 19).

   ii) Similar results were achieved using beetle luciferase, consisting largely of a different amino acid sequence, from the luminous beetle *Pyrophorus plagiophthalamus* (Luc*Ppl*YG). The wild-type *lucPplYG* has 48% nucleotide sequence identity to the wild type *lucPpe2*. The Luc*Ppl*YG mutants were subjected to fewer cycles of directed evolution than the Luc*Ppe2* mutants described herein. Also, in some instances, mutants were selected with less emphasis placed on their relative thermostability.

The most stable clone resulting from this evolution (Luc80-5E5) has a half-life of roughly 3.8 hours at 50°C in solution.

To compensate for a statistical effect caused by the large number of deleterious random mutations expected relative to the beneficial mutations, methods were employed to maximize assay precision and to re-screen previously selected mutations in new permutations. Among the methods for maximizing assay precision were closely controlling culture conditions by using specialized media, reducing growth rates, controlling heat transfer, and analyzing parameters from mid-logarithmic phase growth of the culture. The robotic processes maximized for precision include controlling mixing, heat transfers, and evaporation of samples in the robotic screening process; and normalizing data to spatially distributed control samples. New permutations of the selected mutations were created by a method of DNA shuffling using proof-reading polymerases.

The difficulty in predicting the outcome of the recursive process is exemplified by the variable success with the other characteristics of luciferase that were also selected for. Although the primary focus was on the enzyme thermostability, selection for mutants producing brighter luminescence, with more efficient substrate utilization, and an extended luminescence signal was also attempted. The definitions are given by equations herewith. The selection process was determined by changes relative to the parent clones for each iteration of the recursive process. The amount of the change was whatever was observed during the screening process. The expression of luciferase in *E. coli* was relatively inefficient, for Luc*Ppe2*, compared to Luc +. Other luciferases varied (see Figure 21).

To improve the overall efficiency of substrate utilization, reduction in the composite apparent utilization constant (i.e., Km[ATP+luciferin]) for both luciferin and ATP was sought. Although there was an unexpected systematic change in each utilization constant (Km[ATP], Km[luciferin]), there was little overall change. Finally, the luminescence signal could only be moderately affected without substantially reducing enzyme efficiency. Thus, although the

enzyme thermostability was greatly increased by methods of the present invention, other characteristics of the enzyme were much less affected.

Figures 1-13, 16, 48-53, 60 and 62 present measurements of thermostability of mutant luciferases. Figures 48-53 present other results of the mutant luciferases. Compositions of the invention include luciferases having greater than the natural level of thermostability. Each mutant luciferase is novel, because its individual characteristics have not been reported. Specific luciferases are known by both their protein and gene sequences. Many other luciferases were isolated that have increased, high thermostability, but whose sequences are not known. These luciferases were identified during the directed evolution process, and were recognized as distinct by their enzymological characteristics. The mutant luciferases of the present invention, e.g., Luc90-1B5, can display remarkable and heretofore unrealized thermostability at temperatures ranging from 22°C to at least as high as 60°C.

Other aspects of the invention include methods that incorporate the thermostable luciferases, specifically beetle luciferases having high thermostability, as well as methods to prepare an enzyme, including a luciferase, having one or more desired properties, e.g., resistance to inhibition by a substrate inhibitor, or enhanced enzymological properties. Thus, the invention also provides a method to prepare an enzyme which has at least one enhanced enzymological property. From a population of polynucleotide sequences encoding the enzyme which is derived from a first polynucleotide sequence encoding the enzyme which is subject to mutation, at least one polynucleotide sequence encoding an enzyme which has the enhanced enzymological activity is selected and isolated. In one embodiment, oligonucleotide-mediated mutagenesis is then employed to introduce at least one codon which encodes a consensus amino acid to at least one of the selected, isolated polynucleotide sequences encoding the enzymes to yield a further polynucleotide sequence encoding the enzyme and having the codon which encodes the consensus amino acid, wherein the codon which is introduced is not present in the first polynucleotide sequence.

Production of Luciferases of the Present Invention

The method of making luciferases with increased thermostability is recursive mutagenesis followed by selection. Embodiments of the highly thermostable mutant luciferases of the invention were generated by a reiterative process of random point mutations beginning with a source nucleotide sequence, e.g., the *lucPpe2* [T249M] cDNA. Recombination mutagenesis is a part of the mutagenesis process, along with point mutagenesis. Both recombination mutagenesis and point mutagenesis are performed recursively. Because the mutation process causes recombination of individual mutants in a fashion similar to the recombination of genetic elements during sexual reproduction, the process is sometimes referred to as the sexual polymerase chain reaction (sPCR). See, for instance, Stemmer, U.S. Patent No. 5,605,793, issued February 25, 1997.

Taking the *lucPpe2* cDNA sequence as a starting point, the gene was mutated to yield mutant luciferases which are far more thermostable. A single point mutation to the *lucPpe2* sequence yielded the luciferase whose sequence is depicted as T249M. This mutant is approximately 5 times brighter *in vivo* than that of *lucPpe2*, it was utilized as a template for further mutation. It was also used a baseline for measuring the thermostability of the other mutant luciferases described herein.

Embodiments Of Sequences Of Luciferases Of The Present Invention

Figure 45 shows the amino acid sequence of the Luc*Ppe2* luciferase (T249M). The sequence contains a single mutation at position 249 from T to M (underlined) which distinguishes it from the sequence reported by Leach et al. (1997). This luciferase has a spectral maximum of 552 nm, which is yellow shifted from that of the luciferase of Leach et al. This mutant was selected for use as an original template in some of the Examples because it is approximately 5 times brighter *in vivo*, than the form reported by Leach et al. which allowed for more efficient screening by the assay. These sequences show changes from the starting sequence (T249M) by underlining. Note that "x" in the sequence denotes an ambiguity in the sequence.

Directed Evolution, A Recursive Process

Directed evolution is a recursive process of creating diversity through mutagenesis and screening for desired changes. For enzymological properties that result from the cumulative action of multiple amino acids, directed evolution provides a means to alter these properties. Each step of the process typically produces small changes in enzyme function, but the cumulative effect of many rounds of this process can lead to substantial overall change.

The characteristic, "thermostability" is a candidate for directed evolution because it is determined by the combined action of many of the amino acids making up the enzyme structure. Luminescence output and efficiency of substrate binding of the modified luciferase were also screened. This was to ensure that changes in thermostability did not also produce undesirable changes in other important enzymological properties.

Because the frequency of deleterious mutations is much greater than useful mutations, it is likely that undesirable clones are selected in each screen within the precision limits of the present invention. To compensate for this, the screening strategy incorporated multiple re-screens of the initially selected mutations. However, before re-screening, the selected mutations were "shuffled" to create a library of random intragenetic recombinations. This process allows beneficial mutations among different clones to be recombined together into fewer common coding sequences, and unlinks deleterious mutations to be segregated and omitted. Thus, although essentially the same set of selected mutations was screened again, they were screened under different permutations as a result of the recombination or shuffling.

Although results of each step of the evolutionary process were assayed by quantitative measurements, these measurements were mutually made in cell lysates rather than in purified enzymes. Furthermore, each step only measured changes in enzyme performance relative to the prior step, so global changes in enzyme function were difficult to judge.

Table 1 summarizes the characteristics of various clones obtained using the methods of the invention.

Table 1

Control is Luc39-5B10 at 51°C

Experiment	Clone ID	Luminescence (Li)	Enzyme stability (tau)	Substrate binding (Km)	Signal stability (S)
40	**0a7**	**1.04**	**4.5**	**0.78**	**1**
40	5h4	1.29	1.61	1.16	0.953
40	0c2	1.13	1.54	0.91	0.998
40	5g4	1	1.4	0.85	1
40	6d3	1.02	1.37	0.79	1
40	1g4	1.06	1.28	0.77	0.985
40	1d4	1.69	1.23	0.73	1
40	0h9	1.26	1.21	0.63	0.998
40	2f6	3	1.07	0.49	0.981
40	7d6	3.09	1.058	1.09	1.013
40	5a7	4.3	1.025	0.93	1.008
40	4c8	1	1	0.33	1.004

Experiment	Clone ID	Li	tau	Km	S
41	7h7	0.73	2.4	2.1	0.995
41	5a5	0.77	1.93	2.7	1.002
41	2c12	1.06	1.7	0.91	1.003
41	6e5-	1.16	1.62	1.53	0.997
41	4e5-	1.08	1.37	1.4	1.004
41	6g7	1.3	1.27	1.39	0.999
41	1h4	1.36	1.24	0.56	0.994
41	0c11	4.1	1.23	1.24	0.996
41	2h9	5.3	1.01	0.83	0.986
42	6b10	0.97	3.6	0.97	0.997
42	1c3	0.91	2.1	0.6	0.998
42	7h9	0.8	1.8	0.8	0.982
42	6b2	0.77	1.72	0.8	0.978
42	6d6	0.83	1.7	0.733	0.975
42	4e10-	0.77	1.63	1.8	0.954
42	1b5	0.83	1.41	1.05	0.955
42	6e6-	0.71	1.16	0.89	0.955

42	3a9	0.85	1.3	0.86	0.997
42	6b6	2.7	1.3	0.91	1.02
42	6e9-	1.5	1.27	0.98	1.01
42	3h11	1.73	1.21	0.63	0.985
42	1a2	1.11	1.17	0.77	1.005
42	3f7	0.49	1.16	1.13	0.944
42	1a4	2	1.01	0.76	0.996

Control is Luc40-0A7 at 54°C

Experiment	Clone ID	Li	tau	Km	S
46	**2h3**	**0.86**	**6.4**	**0.37**	**0.96**
46	4a9	0.67	5.7	0.66	0.997
46	2g4	0.65	5.3	0.78	0.96
46	5d12	0.94	4.9	0.94	1.002
46	1h11	1.02	4.8	0.84	0.998
46	5a10	1.23	4.4	0.81	0.9842
46	0a8	1.35	4.3	0.89	1
46	4d3	0.51	3.6	0.65	0.975
46	2a3	1.17	2.9	0.57	0.988
46	3b11	1.39	2.5	0.63	1.02
46	7g12	1.49	2.5	0.91	1.02
46	0g9	1.86	2.25	0.5	0.998
46	7h8	1.07	1.36	0.52	0.99
46	1g8	0.3	1.31	0.72	0.92
46	1d3	1.74	1.13	1.02	1.001
46	0c3	1.68	1.01	0.74	1.01
46	5c11	0.82	1.01	0.6	0.95

Control is Luc46-2H3 at 54°C

Experiment	Clone ID	Li	tau	Km	S
49	6c10	0.57	2.2	0.98	1
49	**7c6**	**1.12**	**1.9**	**0.93**	**1.01**
49	0g12	1	1.58	0.69	1.08
49	7a5	1.08	1.44	1.1	0.99

49	1f6	0.66	1.13	1.04	1.006
49	0b5	0.76	1.07	1.03	0.98
49	4a3	0.94	1.06	0.77	1

Control is Luc49-7C6 at 56°C

Experiment	Clone ID	Li	tau	Km	S
56	2d12	0.97	2.9	0.29	1.006
56	5g10	1.01	2.77	0.64	1.007
56	3d5	1.32	2.25	1.85	1.0

Experiment	Clone ID	Li	tau	Km	S
57	3d1	1.06	2.9	1.05	1.02
57	6g12	1	2.7	0.87	1.004
57	4c1	0.79	2.6	0.93	1.014
57	5f10	0.72	1.9	0.64	1.03
57	1e6-	0.84	1.49	0.984	0.9871
57	1h2	0.94	1.43	0.68	0.991
57	2a6	1.08	1.08	0.89	0.9976

Experiment	Clone ID	Li	tau	Km	S
58	1g6	1.57	8.9	1.78	1.02
58	**0a5**	**1.53**	**8.5**	**1.56**	**1.05**
58	1b1	0.84	8.5	0.6	1.04
58	3g1	1	7.34	0.62	1.006
58	0f3	1.31	6.9	0.57	0.98
58	3e12-	1.06	6.3	0.47	0.996
58	0c7	1.9	4	0.64	1.06
58	0d1	1.03	3.76	0.49	1.03
58	3c7	1.49	3.4	0.55	1.04
58	2a2	1.4	2.2	0.5	1.05
58	2a8	3.2	2	0.81	1.05
58	0f2	2.2	1.92	0.45	1.04

58	1b4	5.1	1.87	1.08	1.09
58	2b3	2.7	1.55	0.57	1.04
58	4g1	4.9	1.2	0.72	1.06

Control is Luc58-0A5 at 58°C

Experiment	Clone ID	Li	tau	Km	S
61	4e9-	1.03	1.84	0.76	1.01
61	1f1	1.02	1.43	0.7	1
61	2e12-	1.56	1.34	0.48	1.003
61	2f2	1.5	1.3	0.32	1.01
61	6b4	1.2	1.26	0.88	0.98
61	4c10	1.46	1.12	1.06	0.99
61	4g11	1.31	1.03	1.43	1.03
61	2f1	1.41	1.02	0.79	0.995
61	2g1	1.3	1	1.17	1

Experiment	Clone ID	Li	tau	Km	S
65	6g12	0.87	2.3	0.73	0.9605
65	1h6	0.84	2.2	1.62	0.9598
65	7f5	1.2	1.56	2.07	1.0087
65	5g5	2.3	1.49	0.45	0.9985
65	7h2	1.56	1.27	0.91	1.0658
65	7b2	1.98	1.16	0.6	0.9289
65	0g9	1.36	1.09	1.46	0.9927
65	6c7	1.48	1.06	0.86	0.9967
65	1e12-	1.59	1.05	1.03	0.9582
65	4e2-	1.21	1.05	1.11	0.943
65	6a10	1.7	1.04	0.93	0.992
65	4b9	1.48	1.04	1.61	1.0009
65	6c1	1.36	1.02	0.72	0.9978

Experiment	Clone ID	Li	tau	Km	S

68	2g6	1.39	3.9	1.17	0.9955
68	4g3	2	2.5	0.27	0.9927
68	5a3	1.04	1.64	0.65	0.8984
68	2b7	1.04	1.64	5.2	0.9237
68	5d10	2.75	1.36	0.73	1.0078
68	7d12	1.85	1.32	0.66	1.0084
68	7b9	1.8	1.19	0.56	1.0052
68	7b3	1.2	1.16	0.55	0.9951
68	1g10	1.48	1.05	1.22	1.0025

Experiment	Clone ID	Li	tau	Km	S
70	2a7	1.94	4.6	0.7	1.0015
70	3d6	3.5	4.2	0.18	1.03
70	4f8	1.87	4.2	0.69	0.9979
70	7h5	2.4	2.6	0.18	1
70	5h6	3.1	2.3	0.6	0.999
70	7d6	3	2.2	2.29	0.9989
70	5a3	3.1	1.5	0.18	1.0058
70	7d2	2.5	1.4	0.66	1.0126
70	3h7	3.2	1.22	0.23	1.002
70	0h5	2.5	1.15	0.36	0.9992
70	0d7	1.86	1	1.83	0.993
70	1g12	2.42	1	0.26	0.965

Experiment	Clone ID	Li	tau	Km	S
71	1d10	1.6	4.5	1.06	1.0065
71	6f11	1.8	4.3	0.98	0.953
71	7h4	3.4	3.6	0.56	1.0045
71	4h3	3.1	3.1	0.42	1.0171
71	1h5	1.31	3.01	1.31	0.9421
71	5e4-	5.4	2.3	0.35	0.994
71	5c1	2.2	2.3	0.89	0.9746
71	0h7	3.6	1.8	0.59	1.0197

71	6h9	23.7	1.71	0.91	1.0064
71	7e3-	5.3	1.7	0.7	1.0028
71	**5d4**	**11.1**	**1.48**	**0.35**	**1.0213**
71	2e3-	4	1.47	0.45	0.9654
71	6h11	17.7	1.15	2.8	1.0064
71	2e10-	3	1.1	0.66	0.9588
71	2g2	4.4	1.01	0.44	1.0046

Control is Luc71-5D4 at 60°C

Experiment	Clone ID	Li	tau	Km	S
72	2g6	0.38	3.1	1.58	1.0052
72	5f12	0.81	1.53	1.02	0.9678
72	0d7	0.76	1.44	1.4	0.9838
72	5c12	0.87	1.43	1.04	0.9718
72	1e1-	1.04	1.41	1.15	0.9956
72	5b12	0.83	1.41	1.02	0.9731
72	0b7	1.11	1.04	0.91	1.0049
72	3b4	0.49	1.03	2.2	0.9581

Experiment	Clone ID	Li	tau	Km	S
73	2h8	0.85	1.9	1.08	1.0123
73	4e6-	0.95	1.76	0.94	0.9939
73	3g8	0.86	1.53	1.04	1
73	1g3	1.7	1.14	0.97	0.9921

Experiment	Clone ID	Li	tau	Km	S
74	2a9	0.96	1.77	0.86	0.999
74	4e10-	0.8	1.36	1.33	0.09897
74	0d5	1.69	1.28	0.61	0.9927
74	6g7	1.75	1.07	1.33	1.0022
74	5d8	0.46	1.06	0.95	0.899
74	5e7-	1.22	1.05	0.87	0.9977

74	6e1-	1.19	1.02	0.96	0.999

Experiment	Clone ID	Li	tau	Km	S
76	6c3	2.3	6.4	1.2	0.9865
76	2a9	0.93	4.7	1.08	0.999
76	3h9	1.26	2.6	1.02	0.9973
76	0b10	1.52	2.4	1.4	0.992
76	0h9	1.71	1.44	1.05	1.018
76	2e9-	0.44	1.15	1.2	0.9318
76	0e10-	1.67	1.1	1.02	1.014
76	0c10	1.13	1.05	1	0.9974
76	3e8-	1.35	1.03	1.1	0.9894
76	0d12	0.69	1	0.92	0.932
76	0f10	0.62	1	1.2	0.9478

Experiment	Clone ID	Li	tau	Km	S
78	1e1-	0.54	8.9	1.15	0.9877
78	0h7	1.4	5	0.97	1.014
78	0a6	1	4.3	1.5	0.9967
78	**0b10**	**1.93**	**2**	**1**	**0.9926**
78	0f11	1.6	2	0.91	0.9905
78	3f1	2.4	1.7	1.09	0.9936
78	2b4	1.97	1.36	0.98	1.0094
78	5b3	3.2	1.19	1.03	0.9735
78	2g12	2.5	1.03	1	1.0134
78	0h2	1.6	1	1.15	1.0168

Control is Luc78-0B10 at 62°C

Experiment	Clone ID	Li	tau	Km	S
82	2g12	0.9811	2.09	0.8851	0.9939
82	4b9	1.0845	1.8419	0.8439	1.0078
82	0d1	0.7622	1.5171	1.11	0.9998

82	3g1	0.8805	1.504	0.9629	0.9927
82	1d1	0.9741	1.4497	0.8936	0.9986
82	1e8-	0.8206	1.4433	0.9876	0.9968
82	0h9	1.1355	1.3626	0.9171	1.0094
82	2c6	1.0931	1.3402	0.9482	1.0022
82	3g9	1.0364	1.251	0.968	1.0009
82	4h8	0.8816	1.1667	0.9165	1.0045
82	0a10	1.0535	1.1128	1.0413	1
82	4g1	1.4305	1.0862	1.1734	1.0059

Experiment	Clone ID	Li	tau	Km	S
84(121)	6h7	0.3755	29.3639	2.3636	0.8905
84(121)	2h9	0.4264	28.7958	1.819	0.904
84(121)	3f7	0.4161	25.3058	1.8079	0.8988
84(121)	2h10	0.9667	14.4658	0.8073	0.9947
84(121)	3a2	0.3329	12.6	2.5444	0.855
84(121)	**3a6**	**1.2299**	**7.2384**	**0.7866**	**1.0046**
84(121)	5b12	1.0535	6.0315	0.7824	1.0056
84(121)	5a7	1.0413	4.9054	0.8864	1.0071
84(121)	3d2	0.2032	4.8	2.4623	0.7973
84(121)	2a9	1.0847	4.7486	0.7746	1.0051
84(121)	5e11-	1.1918	4.0988	0.872	1.008
84(121)	7h2	0.9115	3.9929	0.909	1.0077
84(121)	3b5	1.2014	3.8251	0.7509	1.0086
84(121)	1f8	1.07	3.06	0.8276	1.0093
84(121)	2e2-	1.4356	1.9315	0.7863	1.0175

Control is Luc84-3A6 at 64°C

Experiment	Clone ID	Li	tau	Km	S
85(86)	2a2	0.2266	12.9013	3.326	0.8705
85(86)	**4f12**	**1.1167**	**4.7851**	**0.7439**	**1.0092**
85(86)	4e9-	1.0869	4.4953	0.8539	1.0068
85(86)	1f11	0.6994	4.0976	0.842	1.0124

85(86)	5a4	1.2273	4.09	0.9683	1.0098
85(86)	3e10-	0.8902	3.5342	0.8106	1.0069
85(86)	3e12-	1.0512	3.4883	0.853	1.0054
85(86)	5e4-	0.9562	3.3886	1.0328	1.0069
85(86)	0e6-	0.1494	3.0145	3.6293	0.8269
85(86)	6b1	0.7615	2.5712	0.8695	1.0055
85(86)	6h7	1.0285	2.5401	0.8963	1.0057
85(86)	4b11	0.9816	2.3899	0.7927	1.0063
85(86)	6d7	1.1087	2.0607	0.9042	1.0088
85(86)	2e10-	0.3028	2.0603	1.9649	0.8738
85(86)	2a9	1.448	1.1819	0.9722	1.0046

Control is Luc85-4F12 at 65°C

Experiment	Clone ID	Li	tau	Km	S
88	3c1	1.4439	2.0938	0.9874	0.9976
88	6g1	1.0184	1.2665	1.2184	1.0019
88	3e4-	1.331	1.0996	1.0669	0.9983

Experiment	Clone ID	Li	tau	Km	S
89	1a4	1.2565	2.4796	1.0338	0.997
89	3b1	0.7337	1.9976	0.9628	1.0001
89	2b12	1.0505	1.8496	1.0069	1.0012
89	0b5	1.5671	1.1362	1.0912	0.9995
89	1f1	1.378	1.1018	0.9804	0.996
89	2f1	1.4637	1.0894	0.9189	0.9992

Experiment	Clone ID	Li	tau	Km	S
90	0f1	1.4081	1.3632	1.027	0.9987
90	**1b5**	**1.4743**	**1.1154**	**1.0812**	**1.0011**
90	6g5	1.2756	1.0605	1.0462	1.0012
90	5e6-	1.0556	1.0569	1.1037	1.0011
90	4e3-	1.2934	1.0291	1.0733	1.0002

To evaluate the impact of directed evolution on enzyme function, clones from the beginning, middle and end of the process (Table 2) were purified and analyzed. The clones selected for this analysis were Luc[T249M], Luc49-7C6, and Luc78-0B10. Another clone, Luc90-1B5, created by a subsequent strategy of oligonucleotide-directed mutagenesis and screening was also purified for analysis.

Table 2: Thermostability Of Luciferase Activity At Different Temperatures (Half-Life In Hours)

	*Room Temperature**	*37°C*	*50°C*	*60°C*
Luc[T249M]	110	0.59	0.01	
Luc49-7C6	430	68	31	6.3
Luc78-0B10	3000	220	47	15

* about 25°C

The effect of directed evolution on thermostability was dramatic. At high temperatures, where the parent clone was inactivated almost instantaneously, the mutant enzymes from the related clones showed thermostability over several hours (see also Table 1, and Figures 1-3, 5-8, 11, 13, 50-52 and 60). Even at room temperature, these mutants are several fold more thermostable than the parent enzyme (see also Figures 4, 9-10, 12, 53, and 62). Subsequent analysis of Luc90-1B5 showed this enzyme to be even more thermostable, having a half-life of 27 hours at 65°C when tested under the same buffer conditions (Figure 16A). With some optimization of buffer conditions, this enzyme showed very little activity loss at 65°C over several hours (citrate buffer at pH 6.5; Figure 16A). This luciferase was stable at 22°C over several weeks when incubated at pH 6.5 (Figure 16B). At times over 100 days at 4°C, the mutant enzymes had increased thermostability. At times of less than 15 days at 4°C, the thermostabilities of the mutants Luc49-7C6 and Luc78-0B10 were not distinguishable from the parent enzyme (Figure 49).

Kajiyama and Nakamo (1993) showed that a single amino acid substitution of A at position 217; to either I, L, or V, in the firefly luciferase from *Luciola*

lateralis, resulted in a luciferase having increased thermostability. Substitution with leucine produced a luciferase that maintained 70% of its activity after incubation for 1 hour at 50°C. All of the enzymes of the present invention created through directed evolution, are much more stable than this *L. lateralis* mutant. One clone, Luc90-1B5, maintains 75% activity after 120 hours (5 days) incubation under similar conditions (50°C, 25 mol/L citrate pH 6.5, 150 mmol/L NaCl, 1 mg/mL BSA, 0.1 mmol/L EDTA, 5% glycerol). Interestingly, the Luc*Ppe2* reported by Leach et al. already contains isoleucine at the homologous position described for the *L. lateralis* mutant.

Although thermostability was the characteristic of interest, clones were selected based on the other enzymological parameters in the screens. By selecting clones having greater luminescence expression, mutants were found that yielded greater luminescence intensity in colonies of *E. coli*. However, the process showed little ability to alter the kinetic profile of luminescence by the enzymes. This failure suggests that the ability to support steady-state luminescence is integral to the catalytic mechanism, and is not readily influenced by a cumulative effect of many amino acids.

Substrate binding was screened by measuring an apparent composite K_m (see Example 2) for luciferin and ATP. Although the apparent composite K_m remained relatively constant, later analysis showed that the individual K_m's systematically changed. The K_m for luciferin rose while the K_m for ATP declined (Table 3). The reason for this change is unknown, although it can be speculated that more efficient release of oxyluciferin or luciferin inhibitors could lead to more rapid enzyme turnover.

Each point mutation, on its own, increases (to a greater or lesser extent) the thermostability of the mutant enzyme relative to the wild-type luciferase. The cumulative effect of combining individual point mutations yields mutant luciferases whose thermostability is greatly increased from the wild-type, often on the order of a magnitude or more.

Table 3: Michaelis-Menten Constants for Mutants Created by Directed Evolution

	K_m-luciferin	K_m-ATP
Luc[T249M]	0.32 μM	18 μM
Luc49-7C6	0.99 μM	14 μM
Luc78-0B10	1.6 μM	3.4 μM
Luc90-1B5	2.2 μM	3.0 μM

The following examples illustrate the methods and compositions of the present invention and their embodiments.

EXAMPLE 1

Producing Thermostable Luciferases Of The Present Invention

Mutagenesis Method.

An illustrative mutagenesis strategy is as follows: From the "best" wild-type luciferase clone, that is a clone with increased thermostability and not appreciably diminished values for other parameters, random mutagenesis was performed by three variations of error-prone PCR. From each cycle of random mutagenesis, 18 of the best clones were selected. DNA was prepared from these clones yielding a total of 54 clones. These clones represent new genetic diversity.

These 54 clones were combined and recombination mutagenesis was performed. The 18 best clones from this population were selected.

These 18 clones were combined with the 18 clones of the previous population and recombination mutagenesis was performed. From this screening, a new luciferase population of 18 clones was selected representing 6 groups of functional properties.

In this screening the new mutations of the selected 54 clones, either in their original sequence configurations or in recombinants thereof, were screened a second time. Each mutation was analyzed on the average about 10 times. Of the 90 clones used in the recombination mutagenesis, it was likely that at least 10 were functionally equivalent to the best clone. Thus, the best clone or recombinants thereof should be screened at least 100 times. Since this was greater than the number of clones used in the recombination, there was

significant likelihood of finding productive recombination of the best clone with other clones.

Robotic Processing Methods.

Heat transfers were controlled in the robot process by using thick aluminum at many positions where the 96-well plates were placed by the robotic arm. For example, all shelves in the incubators or refrigerator were constructed from ¼ inch aluminum. One position in particular, located at room temperature, was constructed from a block of aluminum of dimensions 4.5 x 7 x 6.5 inches. When any 96-well plate was moved from a high temperature (e.g., incubators) or low temperature (e.g., refrigerator) to a device at room temperature, it was first placed on the large aluminum block for temperature equilibration. By this means, the entire plate would rapidly reach the new temperature, thus minimizing unequal evaporation for the various wells in the plate due to temperature differences. Heat transfers in a stack of 96-well plates placed in an incubator (e.g., for overnight growth of *E. coli*) were controlled by placing 1 mm thick sheets of aluminum between the plates. This allowed for more efficient heat transfer from the edges of the stack to the center. Mixing in the robotic process was controlled by having the plate placed on a shaker for several second after each reagent addition.

Please refer to Figure 14 for a schematic of the order in which the plates are analyzed and to Figure 15 for a robotic apparatus which can be programmed to perform the following functions:

1. Culture Dilution Method.

A plate with lid (Falcon 3075) containing cells (*E. coli* JM109) is placed on a shaker and mixed for 3-5 minutes.

A plate (with lid) is obtained from a carousel and placed in the reagent dispenser. 180 µl of media (M9 minimal media) is added after removing the lid and placing on the locator near the pipetter. The plate is then placed in the pipetter.

The plate on the shaker is placed in the pipetter, and the lid removed and placed on the locator. Cells are transferred to the new plate using pipetting procedure (see "Dilution of Cells into New Cell Plate").

The lids are replaced onto both plates. The new plate is placed in the refrigerator and the old plate is returned to the carousel.

2. Luminescence Assay Method.

A plate containing cells is retrieved from the carousel and placed on the shaker for 3-5 minutes to fully mix the cells. The cells tend to settle from solution upon standing.

To measure Optical Density (O.D.), the plate is moved from the shaker to the locator near the luminometer; the lid is removed and the plate placed into the luminometer. The O.D. is measured using a 620 nm filter.

When it is finished, the plate is then placed in the refrigerator for storage.

The above steps are completed for all plates before proceeding with subsequent processing.

To prepare a cell lysate, the plate of cells is first retrieved from the refrigerator and mixed on the shaker to resuspend the cells. A new plate from the carousel without a lid is placed in the reagent dispenser and 20 µl of Buffer A is added to each well. This is placed in the pipetting station.

The plate of cells in the shaker is placed in the pipetting station. A daughter plate is prepared using pipetting procedure (see "Pipetting Cells into the Lysis Plate") to prepare a daughter plate of cells.

After pipetting, the new daughter plate is placed on the shaker for mixing.

After mixing, the Lysate Plate is placed into a solid CO_2 freezing station to freeze the samples. The plate is then moved to the thaw block to thaw for 10 minutes.

The plate is then moved to the reagent dispenser to add 175 µl of Buffer B, and then mixed on the shaker for about 15 minutes or more. The combination of the freeze/thaw and Buffer B will cause the cells to lyse.

A new plate with a lid from the carousel is used to prepare the dilution plate from which all assays will be derived. The plate is placed in the reagent dispenser and the lid removed to the locator near the pipetter. 285 µl of Buffer C is added to each well with the reagent dispenser, then the plate is placed in the pipetting station.

The Lysate Plate in the shaker is moved to the pipetting station and pipetting procedure (see "Dilution from Lysis Plate to Incubation Plate") is used. After pipetting, the new daughter plate is placed on the shaker for mixing. The Lysate Plate is discarded.

Two white assay plates (Labsystems #9502887) are obtained from the plate feeder and placed in the pipetter. The incubation plate from the shaker is placed in the pipetter, and the lid removed and placed on the nearby locator. Two daughter plates are made using the pipetting procedure (see "Create Pair of Daughter Plates from Incubation Plate"). Afterwards, the lid is replaced on the parent plate, and the plate is placed in a high temperature incubator. [ranging from 31°C to about 65°C depending on the clone.]

One daughter plate is placed in the luminometer and the 1X assay method is used. After the assay, the plate is placed in the ambient incubator, and the second daughter plate is placed in the luminometer. For the second plate, the 0.02X assay method is used. This plate is discarded, and the first plate is returned from the incubator to the luminometer. The repeat assay method is used (i.e., no reagent is injected). Afterwards, the plate is again returned to the ambient incubator.

The above steps are completed for all plates before proceeding with processing.

To begin the second set of measurements, the plate from the high temperature incubator is placed in the shaker to mix.

The plate in the ambient incubator is returned to the luminometer and the repeat assay method is again used. The plate is returned afterwards to the ambient incubator.

Two white assay plates again are obtained from the plate feeder and placed in the pipetter. The plate on the shaker is placed in the pipetter, and the lid removed and placed on the nearby locator. Two daughter plates are again made using the pipetting procedure (see "Create Pair of Daughter Plates from Incubation Plate"). Afterwards, the lid is replaced on the parent plate, and the plate is returned to the high temperature incubator.

One daughter plate is placed in the luminometer and the 1X assay method is again used. The plate is discarded after the assay. The second daughter plate is

then placed in the luminometer and the 0.06X assay method is used. This plate is also discarded.

The above steps are completed for all plates before proceeding with processing.

In the final set of measurements, the plate from the high temperature incubator is again placed in the shaker to mix.

The plate in the ambient incubator is returned to the luminometer and the repeat assay method is again used. The plate is discarded afterwards.

One white assay plate is taken from the plate feeder and placed in the pipetter. The plate from the shaker is placed in the pipetter, and the lid removed and placed on the nearby locator. One daughter plate is made using the pipetting procedure (see "Create Single Daughter Plate from Incubation Plate"). The lid is replaced on the parent plate and the plate is discarded.

The daughter plate is placed in the luminometer and the 1X assay method is used. The plate is discarded after the assay.

Buffers and Assay Reagents

Buffer A: 325 mM K_2HPO_2; 6.5 mM CDTA; 0.1% Triton X-100

Buffer B: 1X CCLR (Promega E153A); 1.25 mg/ml lysozyme; 0.04% gelatin

Buffer C: 10 mM HEPES; 150 mM NaCl; 1 mg/ml BSA; 5% glycerol; 0.1 mM EDTA

1X Assay reagent: 5 μM Luciferin; 175 μM ATP; 20 mM Tricine, pH 8.0; 0.1 mM EDTA

0.02X Assay reagent: 1:50 dilution of 1X Assay reagent

0.06X Assay reagent: 1:16.7 dilution of 1X Assay reagent

Pipetting Procedures

A. Pipetting Cells Into the Lysis Plate

Non-aseptic procedure using fixed tips

On the pipetter deck:

-place a plate containing approximately 200 μl JM109 cells per well without lid

-Lysate Plate containing 20 μl of Buffer A

Procedure:

1. Move the tips to the washing station and wash with 1 ml.
2. Move to the cell plate and withdraw 60 μl.
3. Move to the Lysate Plate and dispense 45 μl.
4. Repeat steps 1-3 for all 96 samples.
5. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

- Place Lysate Plate onto the shaker.
- Place lid on plate with cells and place on carousel.
- Place Lysate Plate into the CO_2 freezer.

B. Dilution From Lysis Plate to Incubation Plate

On the pipetter deck:

- Lysate Plate containing 240 μl of lysate
- Incubation Plate without lid containing 285 μl of Buffer C

Procedure:

1. Move the tips to the washing station and wash with 0.5 ml.
2. Move to the Lysate Plate and withdraw 30 μl.
3. Move to the Incubation Plate and dispense 15 μl by direct contact with the buffer solution.
4. Repeat steps 1-3 for all 96 samples.
5. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

- Place Incubation Plate on shaker.
- Discard Lysate Plate.

C. Create Pair of Daughter Plates From Incubation Plate

This procedure is done twice

On the pipetter deck:

- Incubation Plate containing 100-300 μl of solution without lid
- Two empty Assay Plates (white)

Procedure:

1. Move the tips to the washing station and wash with 0.5 ml.
2. Move to the Incubation Plate and withdraw 50 µl.
3. Move to the first Assay Plate and dispense 20 µl.
4. Move to the second Assay Plate and dispense 20 µl.
5. Repeat steps 1-4 for all 96 samples.
6. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

1. Replace lid on Incubation Plate.
2. Place Incubation Plate in incubator.
3. Place first Assay Plate in luminometer.
4. Place second Assay Plate on carousel.

D. Create Single Daughter Plate From Incubation Plate

On the pipetter deck:

- Place incubation Plate containing 100-300 µl of solution without lid and
- Empty Assay Plate (white)

Procedure:

1. Move the tips to the washing station and wash with 0.5 ml.
2. Move to the Incubation Plate and withdraw 40 µl.
3. Move to the Assay Plate and dispense 20 µl.
4. Repeat steps 1-3 for all 96 samples.
5. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

- Discard Incubation Plate and lid on Incubation Plate.
- Place Assay Plate in luminometer.

E. Dilution of Cells Into New Cell Plate

Aseptic procedure using fixed tips

On the pipetter deck:

- plate containing approximately 200 µl of cells without lid
- new cell plate containing 180 µl of Growth Medium without lid

Procedure:

1. Move to the cell plate and withdraw 45 µl.
2. Move to the Cell Plate and dispense 20 µl volume by direct liquid-to-liquid transfer.
3. Move to waste reservoir and expel excess cells.
4. Move to isopropanol wash station aspirate isopropanol to sterilize tips.
5. Move to wash station, expel isopropanol and wash tips.
6. Repeat steps 1-4 for all 96 samples.

Post-procedure:

- Replace lid on original plate of cells and place onto carousel.
- Replace lid on new cell plate and place into refrigerator.

Notes:

This procedure is used to prepare the cell plates used in the main analysis procedure. 180 µl of M9 minimal growth medium is added by the reagent dispenser to each of the new cell plates just prior to initiating the pipetting procedure. The dispenser is flushed with 75% isopropanol before priming with medium. The medium also contains selective antibiotics to reduce potential contamination.

Luminometer Procedures

A. 1X Assay Method

1. Place plate into luminometer.
2. Inject 100 µl of 1X Assay reagent.
3. Measure luminescence for 1 to 3 seconds.
4. Repeat for next well.
5. Continue until all wells are measured.

B. 0.02X Assay Method

1. Place plate into luminometer.
2. Inject 100 µl of 0.02X Assay reagent.
3. Measure luminescence for 1 to 3 seconds.
4. Repeat for next well.
5. Continue until all wells are measured.

C. 0.06X Assay Method

1. Place plate into luminometer.
2. Inject 100 µl of 0.06X Assay reagent.
3. Measure luminescence for 1 to 3 seconds.
4. Repeat for next well.
5. Continue until all wells are measured.

D. Repeat Assay

1. Place plate into luminometer.
2. Measure luminescence for 1 to 3 seconds.
3. Repeat for next well.
4. Continue until all wells are measured.

In Vivo Selection Method

Five to seven nitrocellulose disks having 200-500 colonies per disk (1000-3500 colonies total) are screened per 2 microplates (176 clones) (Wood and DeLuca, 1987). The clones are screened at high temperatures using standard screening conditions.

Eight positions in each microplate are reserved from a reference clone using the "best" luciferase (the parent clone for random mutagenesis and codon mutagenesis). The positions of the reserved wells is shown as "X" below.

```
XooooooooooX
oooooooooooo
oooXooooXooo
oooooooooooo
oooooooooooo
oooXooooXooo
oooooooooooo
XooooooooooX
```

The reference clones are made by placing colonies from DNA transformed from the parent clone into the reference wells. To identify these

wells prior to inoculation of the microplate, the wells are marked with a black marking pen on the bottom of each well.

Screening Selection Criteria

The following criteria were used for screening purposes. The temperature chosen for the enzyme stability parameter was such that the parent enzyme would decay 100 to 1000 fold over 10 hours (see Table 1). Criteria 1 is achieved manually; data for criteria 2-6 is generated by robotic analysis. For all criteria, the maximum value as described is selected.

1. ***In vivo* screen**. The brightest clones are selected at a given temperature.
2. **Expression/specific activity**. The value for normalized luminescence is calculated as the ratio of luminescence to optical density. The value is reported as the ratio with the reference value.
3. **Enzyme stability**. Measurements of normalized luminescence of the incubated samples (3 taken over about 15 hours) are fitted to $\ln(L) = \ln(L_0)-(t/\tau)$, where L is normalized luminescence and t is time. τ is a measure of the enzyme stability. The value is reported as the ratio with the reference value, and the correlation coefficients are calculated.
4. **Substrate binding**. Measurements of normalized luminescence with 1X and 0.02X are taken at the initial reading set, and 1X and 0.06X are taken at the 5 hour set. The ratio of the 0.02X:1X and 0.06X:1X gives the relative luminescence at 0.02X and 0.06X concentrations. These values, along with the relative luminescence at 1X (i.e., 1), are fitted to a Lineweaver-Burk plot to yield the Km:app,total for the substrates ATP, luciferin, and CoA. The values are reported as the inverse ratio with the reference value, and the correlation coefficients are calculated.
5. **Signal stability**. The luminescence of the initial 1X luminescent reactions are re-measured 3 additional times over about 15 hours. These values are fitted to $\ln(L) = \ln(L_0)-(t/\tau)$ and the integral over

t (15 hours) is calculated. Signal stability is then calculated as S = (1-int(L)/L_0t)^2. The values are reported as the inverse ratio with the reference value, and the correlation coefficients are calculated.

6. **Composite fitness**. The values of criteria 2 through 5 are combined into a single composite value of fitness (or commercial utility). This value is based on a judgment of the relative importance of the other criteria. This judgment is given below:

Criteria	Relative Value
Enzyme Stability	5
Signal Stability	2
Substrate Binding	2
Expression/Activity	1

The composite, C = Sum(criteria 2-5 weighted by relative value, e.g., more weight is on stability because that was a major goal).

EXAMPLE 2

Software

Organize data into SQL database

Each file created by a luminometer (96 well, Anthos, Austria) represents the data from one microplate. These files are stored in the computer controlling the luminometer, and connected to the database computer by a network link. From each microplate of samples, nine microplates are read by the luminometer (the original microplate for optical density and eight daughter microplates for luminescence).

Ninety files are created in total; each containing data sets for 96 samples. Each data set contains the sample number, time of each measurement relative to the first measurement of the plate, luminometer reading, and background corrected luminometer reading. Other file header information is also given. The time that each microplate is read is also needed for analysis. This can be obtained from the robot log or the file creation time. A naming convention for

the files is used by the robot during file creation that can be recognized by SQL (e.g. YYMMDDPR.DAT where YY is the year, MM is the month, DD is the day, P is the initial plate [0-9], and R is the reading [0-8]).

Data Reduction and Organization

Normalize luminescence data: For each measurement of luminescence in the eight daughter plates, the normalized luminescence is calculated by dividing the relative light units by the optical density of the original plate. If any value of normalized luminescence is less than zero, assign the value of 0.1 sL where sL is the standard deviation for measurements of normalized luminescence.

Calculate relative measurement time: For each normalized luminescence measurement, the time of the measurement is calculated relative to the first measurement of the sample. For example, the times of all luminescence measurements of sample B6 in plate 7 (i.e., 7:B06) are calculated relative to the first reading of 7:B06. This time calculation involves both the time when the plate is read and the relative time of when the sample is read in the plate.

Calculate enzyme stability (τ): For each sample, use linear regression to fit $\ln(L_{1x}) = \ln(L_0)-(t/\tau)$ using the three luminescence measurements with 1X substrate concentrations (Plates 1, 5, 8). Also calculate the regression coefficient.

Calculate substrate binding ($K_{m:app,total}$): Using microplates from the first set of readings (Plates 1 and 2), calculate the $L_{0.2x,rel}$ by dividing measurements made with substrate concentrations of 0.02X by those of 1X. Similarly, calculate the $L_{0.06x,rel}$ using microplates of the second set of readings (Plates 5 and 6), by dividing measurements made with substrate concentrations of 0.06X by those of 1X.

For each sample, use linear regression to fit $1/L = (K_{m:app,total}/L_{max:app})(1/[S])+(1/L_{max:app})$ using

L	[S]
$L_{0.02x,rel}$	0.02
$L_{0.06x,rel}$	0.06
1 ($L_{1x,rel}$)	1

$\bar{K}_{m:app,total}$ is calculated as the slope/intercept. Also calculate the regression coefficient.

Calculate signal stability (S): For each sample, use linear regression to fit $\ln(L) = \ln(L_0)-(t/\tau)$ using the four luminescence measurements of the initial microplate with 1X substrate concentrations (Plates 1, 3, 4, and 7). Also calculate the regression coefficient. From the calculated values of τ and L_0, calculate the integral of luminescence by $\mathrm{int}(L) = \tau L_0 (1-\exp(-t_f/\tau))$, where t_f is the average time of the last measurement (e.g., 15 hours). The signal stability is calculated as $S = (1-\mathrm{int}(L)/L_i t_f)^2$, where L_i is the initial measurement of normalized luminescence with 1x substrate concentration (Plate 1).

[Note: To correct for evaporation, an equation $S = (1+K-\mathrm{int}(L)/L_i t_f)^2$, may be used where $1/K = 2$(relative change of liquid volume at t_f).]

Calculate the reference value surfaces: A three dimensional coordinate system can be defined by using the grid positions of the samples within a microplate as the horizontal coordinates, and the calculated values for the samples (L_i, τ, $K_{m:app,total}$, or S) as the vertical coordinates. This three dimensional system is referred to as a "plate map". A smooth surface in the plate maps representing a reference level can be determined by least squares fit of the values determined for the 8 reference clones in each microplate. For each of the 10 initial microplates of samples, respective reference surfaces are determined for the criteria parameters L_i, τ $K_{m:app,total}$, and S (40 surfaces total).

In the least squares fit, the vertical coordinates (i.e., the criteria parameters) are the dependent variables, the horizontal coordinates are the independent variables. A first order surface (i.e., $z = ax+by+c$) is fitted to the values of the reference clones. After the surface is calculated, the residuals to each reference clone are calculated. If any of these residuals is outside of a given cutoff range, the reference surface is recalculated with omission of the aberrant reference clone.

If a first order surface does not sufficiently represent the values of the reference clones, a restricted second order surface is used (i.e., $z = a(x^2+ky^2)+bx+cy+d$, where k is a constant).

Calculate the reference-normalized values: For the criteria parameter of each sample, a reference-normalized value is determined by calculating the ratio

or inverse ratio with the respective reference value. The reference-normalized values are L_i/L_{ir}, τ/τ_r, $K_{mr}/K_{m:app,total}$, and S_r/S, where reference values are calculated from the equations of the appropriate reference surface.

Calculate the composite scores: For each sample, calculate $C = 5(\tau/\tau_r)+2(S_r/S)+2(K_{mr}/K_{m:app,total})+(L_i/L_{ir})$.

Determine subgroupings: For the criteria parameters L_i, τ, $K_{m:app,total}$, S, and C, delimiting values (i.e., bin sizes) for subgroupings are defined as gL, gτ, gKm, gS, and gC. Starting with the highest values for L_i, τ, or C, or the lowest values of $K_{m:app,total}$ or S, the samples are assigned to bins for each criteria parameter (the first bin being #1, and so on).

Display sorted table of reference-normalized values: Present a table of data for each sample showing in each row the following data:

- sample identification number (e.g., 7:B06)
- composite score (C)
- reference-normalized enzyme stability (τ/τ_r)
- correlation coefficient for enzyme stability
- bin number for enzyme stability
- reference-normalized signal stability (S_r/S)
- correlation coefficient for signal stability
- bin number for signal stability
- reference-normalized substrate binding ($K_{mr}/K_{m:app,total}$)
- correlation coefficient for substrate binding
- bin number for substrate binding
- reference-normalized expression/specific activity (L_i/L_{ir})
- bin number for expression/specific activity

The table is sorted by the composite score (C).

Present sorted table of criteria parameters.

Present a table of data for each sample showing in each row the following data:

- sample identification number
- composite score (C)
- enzyme stability (τ)
- correlation coefficient for enzyme stability

- bin number for enzyme stability
- signal stability (S)
- correlation coefficient for signal stability
- bin number for signal stability
- substrate binding ($K_{m:app,total}$)
- correlation coefficient for substrate binding
- bin number for substrate binding
- expression/specific activity (L_i)
- bin number for expression/specific activity

The table is sorted by the composite score (C); the reference clones are excluded from the table. Same entry coding by standard deviation as described above.

Present sorted table of reference-normalized values

This is the same procedure as the final step of the data reduction procedure. The table will show:

- sample identification number
- composite score (C)
- reference-normalized enzyme stability (τ/τ_r)
- correlation coefficient for enzyme stability
- bin number for enzyme stability
- reference-normalized signal stability (S_r /S)
- correlation coefficient for signal stability
- bin number for signal stability
- reference-normalized substrate binding (K_{mr} /$K_{m:app,total}$)
- correlation coefficient for substrate binding
- bin number for substrate binding
- reference-normalized expression/specific activity (L_i /L_{ir})
- bin number for expression/specific activity

The table is sorted by the composite score (C); the reference clones are excluded from the table. Same entry coding by standard deviation as described above.

Present sorted table of criteria parameters for reference clones

This is the same procedure as described above for criteria parameters, except for only the reference clones. The table will show:

- sample identification number
- composite score (C)
- enzyme stability (τ)
- correlation coefficient for enzyme stability
- bin number for enzyme stability
- signal stability (S)
- correlation coefficient for signal stability
- bin number for signal stability
- substrate binding ($K_{m:app,total}$)
- correlation coefficient for substrate binding
- bin number for substrate binding
- expression/specific activity (L_i)
- bin number for expression/specific activity

The table is sorted by the composite score (C). Same entry coding by standard deviation as described above.

Present sorted table of reference-normalized values

This is the same procedure as described above for reference-normalized values, except for only the reference clones. The table will show:

- sample identification number
- composite score (C)
- reference-normalized enzyme stability (τ/τ_r)
- correlation coefficient for enzyme stability
- bin number for enzyme stability
- reference-normalized signal stability (S_r/S)
- correlation coefficient for signal stability
- bin number for signal stability
- reference-normalized substrate binding ($K_{mr}/K_{m:app,total}$)
- correlation coefficient for substrate binding
- bin number for substrate binding
- reference-normalized expression/specific activity (L_i/L_{ir})

- bin number for expression/specific activity

The table is sorted by the composite score (C). Same entry coding by standard deviation as described above.

Sort table

Any table may be sorted by any entries as primary and secondary key.

Display histogram of table

For any table, a histogram of criteria parameter vs. bin number may be displayed for any criteria parameter.

Display plate map

For any plate, a plate map may be displayed showing a choice of:

- any luminescence or optical density measurement
- L_i
- L_i reference surface
- L_i / L_{ir}
- τ
- τ reference surface
- τ / τ_r
- correlation coefficient of τ
- S
- S reference surface
- S_r / S
- correlation coefficient of S
- $K_{m:app,total}$
- K_m reference surface
- $K_{mr} / K_{m:app,total}$
- correlation coefficient for $K_{m:app,total}$
- composite score (C)

The plate maps are displayed as a three dimensional bar chart. Preferably, the bars representing the reference clones are indicated by color or some other means.

Display drill-down summary of each entry

For L_i, τ, $K_{m:app,total}$, and S, any entry value in a table may be selected to display the luminescence and optical density reading underlying the value

calculation, and a graphical representation of the curve fit where appropriate. Preferably the equations involved and the final result and correlation coefficient will also be displayed.

$\underline{L_i \text{ or } L_i/L_r}$. Display the optical density and luminescence value from the chosen sample in Plate 0 and Plate 1.

$\underline{\tau \text{ or } \tau/\tau_r}$. Display the optical density and luminescence value from the chosen sample in Plate 0, Plate 1, Plate 5, and Plate 8. Display graph of ln(L1X) vs. t, showing data points and best line.

$\underline{S \text{ or } S_r/S}$. Display the optical density and luminescence value from the chosen sample in Plate 0, Plate 1, Plate 3, Plate 4, and Plate 7. Display graph of ln(L) vs. t, showing data points and best line.

$\underline{K_{m:app,total} \text{ or } K_{mr}/K_{m:app,total}}$. Display the optical density and luminescence value from the chosen sample in Plate 0, Plate 1, Plate 2, Plate 5, and Plate 6. Display graph of 1/L vs. 1/[S], showing data points and best line.

EXAMPLE 3

Preparation Of Novel Luciferases

The gene shown in Figure 45 contains a single base pair mutation which encodes an amino acid substitution at position 249, T to M. This clone has a spectral maximum of 552 nm which is yellow shifted from the sequence of Luc. This mutant was selected as an original template because it produces about 5 times brighter luminosity *in vivo* which allowed for more efficient screening.

C-terminus mutagenesis

To eliminate the peroxisome targeting signal (SKL), the L was mutated to a STOP codon and the 3 codons immediately upstream were randomized according to the oligonucleotide mutagenesis procedure described herein. The mutagenic oligonucleotide designed to accomplish this also introduces a unique SpeI site to allow mutant identification without sequencing. The mutants were screened *in vivo* and 13 colonies picked, 12 of which contained the SpeI site.

N-terminus mutagenesis

To test if expression could be improved, the 3 codons immediately downstream from the initiation Met were randomized as described herein. The mutagenic oligo designed to accomplish this also introduces a unique ApaI site

to allow mutant identification without sequencing. Seven clones were selected, and six of the isolated plasmids were confirmed to be mutants.

Shuffling of C- and N-terminus mutants

The C- and N-terminus mutagenesis were performed side-by-side. To combine the N- and C-terminus mutations, selected clones from each mutagenesis experiment were combined with the use of recombination mutagenesis according to the recombination mutagenesis protocol described herein. The shuffled mutants were subcloned into amp^S pRAM backbone and screened in DH5 F'IQ (BRL; Hanahan, 1985). A total of 24 clones were picked, only 4 contained both the N- and C- terminus mutations. These 4 clones were used as templates for randomization of the cysteine positions in the gene.

Mutagenesis to randomize cysteine positions/Random mutagenesis and recombination mutagenesis in the Luc gene

There are 7 cysteine positions in LucPpe2. It is known that these positions are susceptible to oxidation which could cause destabilization of the protein. Seven oligonucleotides were ordered to randomize the cysteine positions.

The oligonucleotides were organized into two groups based upon the conservation of cysteine in other luciferase genes from different families. Group 1 randomizes the conserved cysteine positions C-60, C-80, and C-162. Group 2 randomizes cysteines that are not strictly conserved at positions C-38, C-127, C-221, and C-257.

The four selected templates from the N- and C-terminus mutagenesis were sub-cloned into an ampicillin-sensitive backbone and single-stranded DNA was prepared for each of the templates. These templates were combined in equal amounts and oligonucleotide mutagenesis was completed as described herein. It was determined by plating an aliquot of the mutS transformation prior to overnight incubation that each of the 2 groups contained 2×10^4 independent transformants. MutS-DNA was prepared for the 2 groups and was then transformed into JM109 cells for screening. Mutants from group 1 were screened *in vivo* and picks were made for a full robotic run. Five clones were selected that had improved characteristics. Mutants from group 2 were screened *in vivo* and picks were made for a full robotic run. The temperature incubator on

the robot was set at 33°C for this set of experiments. Ten clones were selected that had improved characteristics. The fifteen best picks from both groups of the cysteine mutagenesis experiments were shuffled together as described herein and 18 of the best clones were selected after robotic processing.

The "best" clone from the above experiment (Luc31-1G8) was selected as a template for subsequent rounds of mutagenesis. (The high temperature robot incubator temperature was set to 42°C.) Another complete round of mutagenesis was completed.

The 18 best clones from the above mutagenesis were picked and clone (Luc39-5B10) was selected as the best clone and was used as a template for another round of mutagenesis. (The high temperature robot incubator temperature was set at 49°C).

After this cycle, 6 of the best clones were selected for sequencing (the nucleotide sequence and inferred amino acid sequence of five of the clones is shown in Figures 22-26 and 27-31, respectively). Based upon the sequence data, nine positions were selected for randomization and seven oligos were designed to cover these positions. Based upon data generated from the robot, it was determined that the best clone from the group of six clones that were sequenced was clone (Luc49-7C6, Figures 22 and 27). The luciferase gene from this clone was sub-cloned into an ampicillin-sensitive pRAM backbone and single stranded DNA was prepared. The randomization of the selected positions was completed according to the oligonucleotide mutagenesis procedure listed herein.

The randomization oligonucleotides were divided into 4 groups, and transformants from these experiments were picked and two robotic runs were completed. Ten clones were selected from the two experiments. (The high temperature robot incubator temperature on robot was set at 56°C).

The best 10 picks from the above two experiments, and the best 18 picks from the previous population of clones were shuffled together (recombination mutagenesis protocol).

The 18 best clones were selected and clone Luc58-0A5 was determined to be the best clone. This clone was then used as a template for another round of mutagenesis. The high temperature robot incubator temperature was set at 58°C.

Clone Luc71-504 was selected as a new lead clone and another round of mutagenesis was completed. Incubator set at 60°C.

The best 18 picks were selected. The nucleotide sequence and inferred amino acid sequence of 4 clones from experiment 78 are shown in Figures 32-35 and 36-39, respectively, and the best clone from this group was determined to be clone Luc78-0B10. The thermostability of clones at various temperatures is presented in the Figures.

EXAMPLE 4

Mutagenesis Strategy from Clone Luc78-0B10 to Luc90-1B5

Twenty-three oligonucleotides were prepared to change 28 positions to consensus. All of the oligonucleotides were tested individually using oligonucleotide directed mutagenesis with single stranded DNA from clone *luc78-0B10* as a template to determine which oligonucleotides gave an improvement in thermostability. Table 4 lists the mutagenic oligonucleotides.

Table 4

Description	OLIGO SYNTHESIS NUMBER	SEQ ID NO.
A17 to T	6215	48
M25 to L	6216	49
S36 to P; remove *Nsi* I site	6217	50
A101 to V, S105 to N	6218	51
I125 to V	6219	52
K139 to Q	6220	53
V145 to I	6221	54
V194 to I	6222	55
V203 to L, S204 to P	6231	56
A216 to V	6232	57
A229 to Q	6233	58
M249 to T (reversion)	**6234***	59
T266 to R, K270 to E	6235	60
E301 to D	6236	61
N333 to P, F334 to G	6237	62
R356 to K	6238	63
I363 to V	6246	64
A393 to P	6247	65
R417 to H	6248	66
G482 to V	6249	67
N492 to T	6250	68
F499 to Y, S501 to A	6251	69
L517 to V	6252	70
F537 to L	6253	71

*Note that oligonucleotide #6234 does not change a consensus position. This oligonucleotide causes a reversion of position 249 to the wild-type Ppe-2 codon. Although reversion of this position was shown to increase thermostability at 62°C, reversion of this position decreased light output.

Three oligonucleotide-directed mutagenesis experiments with clone *luc78-0B10* as a template were completed. The oligonucleotides for these experiments were divided in the following manner:

a. 6215, 6234, 6236, 6248 (found to give increased thermostability)

b. 6215, 6217, 6218, 6219, 6220, 6221, 6222, 6231, 6233, 6234, 6236, 6238, 6247, 6248, 6249, 6251, 6253 (found to be neutral or have increased thermostability).

c. All 23 oligonucleotides.

Selections from the three experiments listed above were screened with the robotic screening procedure (experiment 84, see Table 1) using *luc78-0B10* as a control. Selections from experiment 84 were recombined using the recombination mutagenesis procedure and then screened with the robotic screening procedure (experiment 85).

Single stranded DNA was prepared from three clones, *luc85-3E12*, *luc85-4F12*, *luc85-5A4*. The nucleotide sequence and inferred amino acid sequence of *luc85-4F12* are shown in Figures 40 and 41, respectively. These clones were used as templates for oligonucleotide-directed mutagenesis to improve codon usage. Positions were selected based upon a codon usage table published in Nucleic Acids Research, vol. 18 (supplement) 1990, page 2402. The table below lists oligonucleotides that were used to improve codon usage in *E. coli.*

Table 5

Description	Oligo Synthesis #	SEQ ID NO.
L7(tta-ctg), remove *Apa* I site	6258	72
L29(tta-ctg)	6259	73
T42(aca-acc)	6260	74
L51, L56(tta-ctg), L58(ttg-ctg)	6261	75
L71(tta-ctg)	6262	76
L85(ttg-ctg)	6263	77
L95(ttg-ctg), L97(ctt-ctg)	6273	78
L113, L117(tta-ctg)	6274	79
L151, L153(tta-ctg)	6275	80
L163(ctc-ctg)	6276	81
R187(cga-cgt)	6277	82
L237(tta-ctg)	6279	83
R260(cga-cgc)	6280	84
L285, L290(tta-ctg), L286(ctt-ctg)	6281	85
L308(tta-ctg)	6282	86
L318(tta-ctg)	6283	87
L341(tta-ctg), T342(aca-acc)	6284	88
L380(ttg-ctg)	6285	89
L439(tta-ctg)	6286	90
L456(ctc-ctg), L457(tta-ctg)	6293	91
T506(aca-acc), L510(cta-ctg)	6305	92
R530(aga-cgt)	6306	93

In the first experiment, the three templates listed above from experiment 85 were combined and used as a templates for oligonucleotide-directed mutagenesis. All of the oligonucleotides were combined in one experiment and clones resulting from oligonucleotide-directed mutagenesis were screened using the robotic screening procedure as experiment 88. There were a low percentage of luminescent colonies that resulted from this experiment, so another oligonucleotide-directed mutagenesis experiment was completed in which the oligonucleotides were combined in the following groups:

a. 6258, 6273, 6280, 6286

b. 6259, 6274, 6281, 6293

c. 6260, 6275, 6282, 6294

d. 6261, 6276, 6283, 6305

e. 6262, 6277, 6284, 9306

f. 6263, 6279, 6285

It was discovered that samples from group b had a low number of luminescent colonies, and it was hypothesized that one of the oligonucleotides in group b was causing problems. Selections were made from all of the experiments with the exception of experiment b. Samples were then run through the robotic screening procedure (experiment 89). Selections from experiments 88 and 89 were shuffled together with the recombination mutagenesis protocol and were then screened with the robotic screening procedure (experiment 90).

Materials and Methods

A. Mutagenesis Protocol

The mutant luciferases disclosed herein were produced via random mutagenesis with subsequent *in vivo* screening of the mutated genes for a plurality of characteristics including light output and thermostability of the encoded luciferase gene product. The mutagenesis was achieved by generally following a three-step method:

1. Creating genetic diversity through random mutagenesis. Here, error-prone PCR of a starting sequence was used to create point mutations in the nucleotide sequence. Because error-prone PCR yields almost exclusively single point mutations in a DNA sequence, a theoretical maximum of 7 amino acid changes are possible per nucleotide mutation. In practice, however, approximately 6.1 amino acid changes per nucleotide is achievable. For the 550 amino acids in luciferase, approximately 3300 mutants are possible through point mutagenesis.

2. Consolidating single point mutations through recombination mutagenesis. The genetic diversity created by the initial mutagenesis is recombined into a smaller number of clones by sPCR This process not only reduces the number of mutant clones, but because the rate of mutagenesis is high, the probability of linkage to negative mutations is significant. Recombination mutagenesis unlinks positive mutations from

negative mutations. The mutations are "re-linked" into new genes by recombination mutagenesis to yield the new permutations. Then, after re-screening the recombination mutants, the genetic permutations that have the "negative mutations" are eliminated by not being selected. Recombination mutagenesis also serves as a secondary screen of the initial mutants prepared by error-prone PCR.

3. <u>Broadening genetic diversity through random mutagenesis of selected codons</u>. Because random point mutagenesis can only achieve a limited number of amino acid substitutions, complete randomization of selected codons is achieved by oligonucleotides mutagenesis. The codons to be mutated are selected from the results of the preceding mutagenesis processes on the assumption that for any given beneficial substitution, other alternative amino acid substitutions at the same positions may produce even greater benefits. The positions to be mutated are identified by DNA sequencing of selected clones.

B. <u>Initial mutagenesis experiments</u>

Both the N-terminus and the C-terminus of the starting sequence were modified by oligonucleotide-directed mutagenesis to optimize expression and remove the peroxisomal targeting sequence. At the N-terminus, nine bases downstream of the initiation codon were randomized. At the C-terminus, nine bases upstream of the termination codon were randomized. Mutants were analyzed using an *in vivo* screen, resulting in no significant change in expression.

Six clones from this screen were pooled, and used to mutate the codons for seven cysteines. These codons were randomized using oligonucleotide-directed mutagenesis, and the mutants were screened using the robotic screening procedure. From this screen, fifteen clones were selected for directed evolution.

C. <u>Generating and Testing Clones</u>

Several very powerful and widely known protocols are used to generate and test the clones of the present invention. Unless noted otherwise, these laboratory procedures are well known to one of skill in the art. Particularly noted as being well known to the skilled practitioner is the polymerase chain

reaction (PCR) devised by Mullis and various modifications to the standard PCR protocol (error-prone PCR, sPCR, and the like), DNA sequencing by any method (Sanger or Maxxam & Gilbert's methodology), amino acid sequencing by any method (e.g., the Edman degradation), and electrophoretic separation of polynucleotides and polypeptides/proteins.

D. Vector Design

A preferred vector (pRAM) (see Figure 20) used for the mutagenesis procedure contains several unique features that allow for the mutagenesis strategy to work efficiently:

The pRAM vector contains a filamentous phage origin, f1, which is necessary for the production of single-stranded DNA.

Two SfiI sites flank the gene. These sites were designed by so that the gene to be subcloned can only be inserted in the proper orientation.

The vector contains a *tac* promoter.

Templates to be used for oligonucleotide mutagenesis contain a 4 base-pair deletion in the *bla* gene which makes the vector ampicillin-sensitive. The oligonucleotide mutagenesis procedure uses a mutant oligonucleotide as well as an ampicillin repair oligonucleotide that restores function to the *bla* gene. This allows for the selection of a high percentage of mutants. (If selection is not used, it is difficult to obtain a high percentage of mutants.)

E. Uses of Luciferases

The mutant luciferases of the present invention are suitable for use in any application for which previously known luciferases were used, including the following:

ATP Assays. The greater enzyme stability means that reagents designed for detection of ATP have a greater shelf-life and operational-life at higher temperatures (e.g., room temperature). Therefore, a method of detecting ATP using luciferases with increased thermostability is novel and useful.

Luminescent labels for nucleic acids, proteins, or other molecules. Analogous to advantages of the luciferases of the present invention for ATP assays, their greater shelf-life and operational-life is a benefit to the reliability

and reproducibility of luminescent labels. This is particularly advantageous for labeling nucleic acids in hybridization procedures where hybridization temperatures can be relatively high (e.g., greater than 40°C). Therefore, a method of labeling nucleic acids, proteins, or other molecules using luciferases of the present invention is novel and useful.

Genetic reporter. In the widespread application of luciferase as a genetic reporter, where detection of the reporter is used to infer the presence of another gene or process of interest, the increased thermostability of the luciferases provides less temperature dependence of its expression in living cells and in cell-free translations and transcription/translation systems. Therefore, a method using the luciferases of the present invention as genetic reporters is novel and useful.

Enzyme immobilization. Enzymes in close proximity to physical surfaces can be denatured by their interaction with that surface. The high density immobilization of luciferases onto a surface to provide strong localized luminescence is improved by using thermostable luciferases. Therefore, a method of immobilizing luciferases onto a solid surface using luciferases of the present invention is novel and useful.

Hybrid proteins. Hybrid proteins made by genetic fusion genes encoding luciferases and of other genes, or through a chemical coupling process, benefit by having a greater shelf-life and operational-life. Therefore, a method of producing hybrid proteins through genetic means or chemical coupling using the luciferases of the present invention is novel and useful.

High temperature reactions. The light intensity of a luciferase reaction increases with temperature until the luciferase begins to denature. Because the use of thermostable luciferases allows for use at greater reaction temperatures, the luciferases of the present invention are novel and useful for performing high temperature reactions.

Luminescent solutions. Luminescence has many general uses, including educational, demonstrational, and entertainment purposes. These applications benefit from having enzymes with greater shelf-life and operational-life. Therefore, a method of making luminescent solutions using the luciferases of the present invention is novel and useful.

F. Firefly luciferase

The firefly luciferase gene chosen for directed evolution was Luc*Ppe2* isolated from *Photuris pennsylvanica*. The luciferase was cloned from fireflies collected in Maryland by Wood et al. and later was independently cloned by Dr. Leach using fireflies collected in Oklahoma (Ye et al., 1997). A mutant of this luciferase (T249M) was made by Wood et al. and used in the present invention because it produced approximately 5-fold more light when expressed in colonies of *E. coli*.

Overview of Evolution Process: Directed evolution was achieved through a recursive process, each step consisting of multiple cycles of 1) creating mutational libraries of firefly luciferase followed by 2) screening the libraries to identify new mutant clones having a plurality of desired enzymological characteristics.

To begin the process, three mutational libraries were created using error-prone PCR (Fromant *et al.,* 1995). Each library was screened first by visual evaluation of luminescence in colonies of *E. coli* (Wood and De Luca, 1987), and then by quantitative measurements of enzymological properties in *E. coli* cell lysates. Approximately 10,000 colonies were examined in the visual screen, from which 704 were selected for quantitative analysis. From each quantitative screen 18 clones were selected. The three sets of 18 clones each were pooled together, and a new mutational library was created using DNA shuffling to generate intragenetic recombinations (sPCR; Stemmer, 1994). The results were screened to yield another set of 18 clones. The entire process was completed by combining this set of 18 clones with 18 clones from the previous round of evolution, creating another mutational library by DNA shuffling, and screening as before.

Screening method: In the qualitative visual screen, colonies were selected only for their ability to sustain relatively bright luminescence. The thermal stability of the luciferase within the colonies of *E. coli* was progressively challenged in successive rounds of evolution by increasing the temperature of the screen. The selected colonies were inoculated into wells of 96-well plates each containing 200 μl of growth medium.

In the quantitative screens, lysates of the *E. coli* cultures were measured for 1) luminescence activity, 2) enzyme stability, 3) sustained enzymatic turnover, and 4) substrate binding.

"Luminescence activity" was measured as the ratio of luminescence intensity to the optical density of the cell culture.

"Enzyme stability" was determined by the rate of activity loss from cell lysates over 10 hours. In successive rounds of evolution the incubation temperature of the lysates was increased.

"Sustained enzymatic turnover" was determined by the rate of luminescence loss of a signal enzymatic reaction over 10 hours at room temperature.

"Substrate binding" was determined by the relative activity of the lysate when assayed with diluted substrate mixtures. Of these four parameters, the highest priority for selection was placed on thermostability.

<u>Robotic Automation</u>: Robotic automation was used in the quantitative screens to accurately perform the large number of required quantitative assays on the cultured cells. Overnight cultures were first diluted into fresh medium and grown for 3 hours to produce cultures in mid-log phase growth. The optical densities of each culture was then measured, and aliquots of the cultures were lysed by freeze/thaw and lysozyme. The resulting lysates were further diluted before analysis and incubated at elevated temperatures. Luminescence was measured from aliquots of the diluted lysates, taken at various times, and measured under various conditions as prescribed by the analytical method (see Example 2). Computer analysis of this data yielded the quantitative selection criteria described herein.

<u>Summary of evolutionary progression</u>: After mutagenesis of the N- and C-termini, and randomization of the cysteine codons, a pool of 15 clones was subjected to two rounds of directed evolution as described herein. Five of the 18 clones resulting from this process were sequenced to identify mutations. One of these clones designated, Luc49-7C6, was chosen for more detailed analysis and further mutagenesis. This clone contained 14 new amino acid substitutions compared to the luciferase Luc[T249M].

To assess the potential for other amino acid replacements at the sites of these substitutions, oligonucleotide-directed mutagenesis was used to randomize these codons. The resulting clones were screened as described herein, and 18 selected clones were used to initiate two new rounds of directed evolution. Of the 18 clones resulting from this second set of rounds, the clone designated Luc78-0B10 was chosen for additional study and mutagenesis. This clone encoded a luciferase that contained 23 new amino acid substitutions compared to Luc[T249M].

Using oligonucleotide directed mutagenesis with Luc78-0B10 as the template, codons were selected for substitution to consensus amino acids previously known among beetle luciferases. Selections from this mutagenesis experiment were shuffled together and three clones, determined to be the most stable were then used as templates for oligonucleotide mutagenesis to improve codon usage in *E. coli*. A clone designated Luc90-1B5 selected from this experiment, contained 34 amino acid substitutions relative to Luc[T249M] (see Figures 42 and 43 for the nucleotide sequence and inferred amino acid sequence of *luc90-1B5*, and Figures 44 and 45 for the nucleotide sequence encoding and the inferred amino acid sequence of Luc[T249M]). Out of 25 codons selected for change to consensus amino acids, 11 were replaced in the clone designated Luc90-1B5. Only five out of the 30 positions that were selected for improved codon usage were substituted and had little effect on enzyme expression.

<u>Protein purification</u>: Four mutants that are described herein (Luc[T249M], Luc49-7C6, Luc78-0B10, and Luc90-1B5) were purified using a previously published procedure (Hastings et al., 1996).

<u>Enzymological characterization</u>: Purified proteins were diluted in 25 mmol/L HEPES pH 7.8, 150 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mg/ml BSA. Enzyme stability was determined from diluted proteins incubated at different temperatures, and aliquots were removed at different time points. A linear regression of the natural log of the luminescence and time was calculated. Half-life was calculated as the ln(0.5)/slope of the regression.

G. PCR Mutagenesis Protocol (Random Mutagenesis)

PCR mutagenesis reactions

1. Prepare plasmid DNA from a vector containing the gene of interest, estimate DNA concentration from a gel.

2. Set up two 50 µl reactions per group:

There are three groups of mutagenic conditions using different skewed nucleotide concentrations.

The conditions listed herein yield in the range of from 8-10% wild-type Luc colonies after subcloning phenotypic for each generated parent clone. The rate of mutagenesis is estimated by the number of luminescent colonies that are present after mutagenesis. Based upon results of clones mutated in the range of 8-10%, it was determined that this level of mutagenesis produces on average approximately 2-3 amino acid changes per gene. If the mutagenesis rate is selected so that on average there is one amino acid change per gene, then on average 50% of the clones will have no mutations. (Bowie et al., 1990).

For the master mix: add all components (see Table 6) except polymerase, vortex, spin briefly, add polymerase, and mix gently.

Table 6

Component	A to T/T to A	A to C/T to G	G to A/C to T
dATP	0.3 mM	0.1 mM	0.25 mM
dCTP	2.75 mM	4 mM	1 mM
dGTP	0.06 mM	0.02 mM	0.05 mM
dTTP	0.625 mM	0.3 mM	0.6 mM
++pRAMtailUP	0.4 pmol/µl	0.4 pmol/µl	0.4 pmol/µl
++pRAMtailDN	0.4 pmol/µl	0.4 pmol/µl	0.4 pmol/µl
*Taq Polymerase	1 U/µl	1 U/µl	1 U/µl
°$MgCl_2$	6.77 mM	5.12 mM	2.7 mM
°$MnCl_2$	0.5 mM	0.5 mM	0.3 mM
DNA	50 ng total	50 ng total	50 ng total
10X PCR buffer	1X	1X	1X
Autoclaved nanopure water	To 50 µl	To 50 µl	To 50 µl

* Taq Polymerase is purchased from Perkin Elmer (N808-0101).

° $MnCl_2$ and $MgCl_2$ are made fresh from 1 M stocks. The stocks are filter sterilized and mixed with sterile water to make the 10 mM and 25 mM stocks which are then stored in Polystyrene Nalgene containers at 4°C.

++ pRAMtailUP: 5'-gtactgagacgacgccagcccaagcttaggcctgagtg-3' (SEQ ID NO:38); pRAMtailDN: 5'-ggcatgagcgtgaactgactgaactagcggccgccgag-3'(SEQ ID NO:39)

<u>10X PCR polymerase buffer</u>:

- 100 mM Tris-HCl pH 8.4 from 1 M stock
- 500 mM Kcl
- Primers are diluted from a 1 nmol/µl stock to a 20 pmol/µl working stock.

Cycle in thermal cycler: 94°C for 1 minute (94°C for 1 minute, 72°C for 10 minutes) 10X

3. Purify reaction products with Wizard PCR purification kit (Promega Corporation, Madison, Wisconsin, part#A718c):
   - transfer PCR reaction into a new tube containing Promega 100 µl Direct Purification buffer (Promega part#A724a)
   - add 1 ml of Wizard PCR Purification Resin (Promega part#A718c) Promega and incubate at room temperature for 1 minute
   - pull resin though Wizard minicolumn
   - wash with 80% ethanol
   - spin in microcentrifuge to remove excess ethanol
   - elute into 50 µl sterile nanopure water (allow water to remain on column for at least 1 minute)

Amplification[1] Of Mutagenesis Reaction

1. Set up five 50 µl reactions (see Table 7) per group.

Table 7

Components	Concentration	Amount in 50 µl	Final concentration
dATP	10 mM	1 µl	0.2 mM
dCTP	10 mM	1 µl	0.2 mM
dGTP	10 mM	1 µl	0.2 mM
dTTP	10 mM	1 µl	0.2 mM
+pRAM18UP	20 pmol/µl	1 µl	0.4 pmol/µl
+pRAM19DN	20 pmol/µl	1 µl	0.4 pmol/µl
Pfu polymerase	2 U/ul	1 µl	0.04 µ/µL
°10X buffer	10X	5 µl	1X
DNA		10 µl	
Water		24.6 µl	

- To master mix: add all components, except polymerase, vortex, spin briefly, add polymerase, mix gently.

° 10X reaction buffer for Native Pfu polymerase contains 20 mM $MgCl_2$, so no additional $MgCl_2$ needs to be added

+ primers:

pRAM18UP –5'-gtactgagacgacgccag-3' (SEQ ID NO:40)

pRAM19DN –5'-ggcatgagcgtgaactgac-3' (SEQ ID NO:41)

Cycling conditions: 94°C for 30 seconds (94°C for 20 seconds, 65°C for 1 minute, 72°C for 3 minutes) 25X (Perkin-Elmer Gene Amp® PCR System 2400)

2. Load 1 µl on a gel to check amplification products

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[1] This amplification step with Pfu polymerase was incorporated for 2 reasons: (a) To increase DNA yields for the production of large numbers of transformants. (b) To reduce the amount of template DNA that is carried over from the mutagenic PCR reaction: (Primers for the second amplification reaction are nested within the mutagenic primers. The mutagenic primers were designed with non-specific tails of 11 and 12 bases respectively for the upstream and downstream primers. The nested primers will amplify DNA that was previously amplified with the mutagenic primers, but cannot amplify pRAM template DNA.)

3. Purify amplification reaction products with Wizard PCR purification kit (Promega Corporation, part#A718c):
   - transfer PCR reaction into a new tube containing 100 µl Direct Purification buffer (Promega, Part#A724a)
   - add 1 ml of Wizard PCR Purification Resin (Promega Part#A718c) and incubate at room temperature for 1 min
   - pull resin though Wizard minicolumn
   - wash with 80% Ethanol
   - spin in microcentrifuge to remove excess Ethanol
   - elute with 88 µl sterile nanopure water (allow water to remain on column for at least 1 min)

Subcloning of amplified PCR mutagenesis products

1. Digest the DNA with *Sfi* I as follows:
   - 2 µl *Sfi* I (Promega Part #R639a)
   - 10 µl 10X buffer B (Promega Part #R002a)
   - 88 µl of DNA from Wizard PCR prep (see step 3 above)
   - mix components and overlay with 2 drops of mineral oil; incubate at 50°C for 1 hour
2. Remove salts and *Sfi* I ends with Wizard PCR purification as described herein, and elute into 50 µl sterile nanopure water
3. Ligation into pRAM (+/r) backbone (set up 4 ligations per group):
   - 0.025 pmol pRAM backbone
   - 0.05 pmol insert (usually in the range of 6 to 12 µl of insert)
   - 1 µl of T4 DNA Ligase (Promega part M180a)
   - 2 µl of 10X ligase buffer (Promega part C126b, divide into 25 µl aliquots, do not freeze/thaw more than twice)
   - water to 20 µl
   - ligate for 2 hours at room temperature
   - heat reactions for 15 minutes at 70°C to inactivate ligase

Transformation and plating

1. Butanol precipitate samples to remove excess salts (n-Butanol from Sigma, St. Louis, Missouri, part #BT-105):

(if ethanol precipitation is used instead of butanol, a wash with 70% ethanol as needed. Excess salt will cause arcing during the electroporation which causes the reaction to fail.)

- add water to 50 μl
- add 500 μl of n-butanol
- mix until butanol/ligation mix is clear and then spin for 20 min at room temperature
- drain butanol into waste container in fume hood
- resuspend in 12 μl water, spin 30 sec at full speed

2. Preparation of cell/DNA mix (set up 4 transformations plus one with reference clone DNA):

- while DNA is precipitating, place electroporation cuvettes on ice
- fill 15 ml Falcon snap-cap tubes with 3 ml S.O.C. medium and place on ice
- thaw JM109 electrocompetent cells on ice (50 μl per ligation reaction)
- pipette 10 μl of the bottom layer from step 1 (or 0.5 μl ref.clone DNA) into competent cells

(small amounts of butanol carry-over do not adversely effect the transformation efficiency)

- place cell/DNA mix on ice

3. Electroporation:

- carry tubes, cuvettes, and cell/DNA mix on ice to electroporation device
- pipette cell-DNA mix into a cuvette and zap. Instrument settings:

Cuvette gap: 0.2 cm
Voltage: 2.5 kV
Capacitance: 25 μF
Resistance: 200 Ohms
Time constant: 4.5 msec

- pipette 1 ml SOC (contains KCl; media prep #KCLM) into cuvette, quickly pour into recovery tube (transformation efficiency is reduced if cells are allowed to sit in cuvette)
- place the recovery tube on ice until all samples are processed
- allow the cells to recover at 37°C for 30-60 minutes
- plate on LB + amp plates with nitrocellulose filters

(# of colonies is about 20% higher if cells recover 60 minutes, possibly due to cell replication.)

(Best colony density for screening is 500 per plate. For the current batch of cells plate about 500 to 750 μl)

H. Recombination Mutagenesis Protocol or DNA shuffling

DNase I digestion of plasmid DNA

1. Prepare 2% low melting point gel
   - use 0.8 g agarose in 40 ml (NuSieve #50082)
   - use large prep comb
   - make sure it is solidified prior to digesting
2. Prepare 4 μg of pooled plasmid DNA for digest
3. Prepare 1 U/μl DNase dilution on ice according to the table below:

Table 8

DNase I⁺	0.74 μl
10X DnaseI buffer	10 μl
1% gelatin*	10 μl
Water to 100 μl	

⁺ DNase I from Sigma (D5791)

* Gelatin was added to keep the DNase I from sticking to the walls of the tubes. This dilution can be kept on ice for at least 30 min without loss in activity.

4. Digest (set up at room temperature):

   prepare two digests with 1.0 U and 1.5 U DNase I per 100 μl reaction:

- 10 µl of 10X DNase I buffer (500 mM Tris, 10 mM $MgCl_2$ pH 7.8)
- x µl DNA (2 µg of pooled plasmid DNA from step 2)
- 1 or 1.5 µl of the 1 U/µl enzyme dilution
- sterile nanopure water to 100 µl
- incubate at room temperature for 10 minutes
- stop reaction by addition of 1 µl of 100 mM CDTA

Purification from agarose gel

1. Run DNase digested fragments on gel
   - add 10 µl of 10X loading buffer to each DNase I digest
   - load all on a 2% Low melting point agarose gel
   - run about 30 min at 120-150 V
   - load pGEM DNA marker in middle lane
2. Isolate fragments
   - cut out agarose slice containing fragments in the size range of 600-1000 bp using a razor blade
   - cut into pieces that weigh about 0.3 g
   - melt the gel slices at 70°C
   - add 300 µl of Phenol (NaCl/Tris equilibrated) to the melted agarose, vortex for about 1 minute at max speed
   - spin for 10 min at 4°C
   - remove the top layer into a tube containing an equal volume of Phenol/Chloroform/Isoamyl (saturated with 300 mM NaCl /100 mM Tris pH 8.0), vortex and centrifuge for 5 minutes at RT
   - remove the top layer into a tube containing chloroform and vortex and centrifuge.
   - remove the top layer into a tube with 2 vol. of 95% cold Ethanol; place in -70°C freezer for 10 min (no additional salts are needed because of the High Salt Phenol)
   - spin at 4°C for 15 minutes.
   - wash with 70% Ethanol, drain and air dry for ~10 min
   - resuspend in 25 to 50 µl of sterile nanopure water

- store at -70°C until ready for use

Assembly reaction

Set up 4 reactions (see Table 9) and pool when completed.

Table 9

Component	Concentration	Amount in µl	Final concentration
dATP	10 mM	1	200 µM
dCTP	10 mM	1	200 µM
dGTP	10 mM	1	200 µM
dTTP	10 mM	1	200 µM
DNA*	1-10 ng	5	
Tli	3 U/µl	0.4	0.24 U/µl
10X Thermo buffer	10X	5	1X
$MgCl_2$	25 mM	4	2 mM
gelatin	1%	5	0.1%
water		To 50 µl	

* Because the DNA used for this reaction has been fragmented, it is difficult to estimate a concentration. The easiest way is to load 5 µl of the DNaseI digested DNA to an agarose gel and run the gel until the dye enters the wells (1-2 min). Fragments from a typical 2 µg DNA digest which were resuspended in 100 µl of water give a DNA concentration of about 1 to 10 ng/µl.

Cycling conditions: 94°C for 30 seconds (94°C for 20 seconds, 65°C for 1 minute, 72°C for 2 minutes) 25X

Amplification of assembly

Usually 5 amplification reactions (see Table 10) will produce enough DNA for a full 8 plate robotic run.

Table 10

Component	Concentration	Amount in µl	Final concentration
dATP	10 mM	1	200 µM
dCTP	10 mM	1	200 µM
dGTP	10 mM	1	200 µM
dTTP	10 mM	1	200 µM
pRAMtailUP*	20 pmol/µl	2	0.8 pmol/µl
pRAMtailDN*	20 pmol/µl	2	0.8 pmol/µl
Pfu native polymerase+	2 U/µl	1	0.04 U/µl
10X native Pfu buffer°	1X	5	1X
DNA	1-10 ng	5	
water		water to 50 µl	

* Note that the concentration of primers is twice as high as in a typical amplification reaction.

° The Pfu 10X buffer contains 20 mM $MgCl_2$, so it is not necessary to add $MgCl_2$.

\+ Pfu polymerase is ordered from Stratagene part #600135. Cycling conditions: 94°C for 30 seconds (94°C for 20 seconds, 65°C for 1 minute, 72°C for 3 minutes) 25x

Subcloning of assembly amplification

Purify amplification products with Wizard PCR purification:

- pool 5 amplification reactions
- transfer into a new tube that contains 100 µl of Direct Purification buffer
- add 1 ml of Wizard PCR Purification Resin, incubate at RT for 1 minute
- pull Resin though Wizard minicolumn
- wash with 80% ethanol and spin in microcentrifuge to remove excess ethanol
- elute with 88 µl of sterile nanopure water (allow water to remain on column for at least 1 minute)

2. Digest with *Sfi* I:

- 2 µl *Sfi* I
- 10 µl 10X buffer B
- 88 µl of DNA from Wizard PCR prep

- mix components and overlay with 2 drops of mineral oil; incubate at 50°C for 1 hour

3. Band isolation:

Sometimes after amplification of the assembly reaction a band that is smaller than the gene-sized fragment is produced. This small fragment has been shown to subclone about 10-fold more frequently than the gene sized fragment if the sample is not band isolated. When this contaminating band is present, it is necessary to band isolate after *Sfi* I digestion.

- load the DNA to a 0.7% agarose gel
- band isolate and purify with the Gene Clean kit from Bio 101
- elute DNA with 50 µl sterile nanopure water, check concentration on gel (This type of purification with standard agarose produced the highest number of transformants after subcloning. Other methods tried: Low melt with Phenol chloroform, Gene clean with low melt, Wizard PCR resin with standard agarose, Pierce Xtreme spin column with Low melt (did not work with standard agarose)).

4. Ligate into pRAM [+/r] backbone: (See ligation and transformation protocol above)

Large scale preparation of pRAM backbone

1. Streak an LB amp plate with pRAMMCS [+/r] (This vector contains a synthetic insert with a *Sac* II site in place of a gene. This vector contains the new ribosome binding site, but it will be cut out when the vector is digested with *Sfi* I.
2. Prepare a 10 ml overnight culture in LB supplemented with amp.
3. The next day inoculate 1 L of LB supplemented with amp and grow for 16-20 hours.
4. Purify the DNA with the Wizard Maxi Prep kit. (Promega #A7270) (use 4 preps for 1 L of cells)

5. Digest the Plasmid with *Sfi* I. (Use 5 U per microgram) Overlay with mineral oil and digest for at least two hours.
6. Ethanol precipitate to remove salts. Resuspend in water.
7. Digest with *Sac* II for 2 hours. (Keep digest volume to 2 ml or less). It is possible that part of the plasmid could be partially digested. If the vector is cut with an enzyme that is internal to the two *Sfi* I sites, it will keep the partially digested fragments from joining in a ligation reaction.
8. Load entire digest onto a column (see 9). The volume of the sample load should not be more than 2 ml. If it is it will be necessary to ethanol precipitate.
9. The column contains Sephacryl S-1000 and is stored with 20% ethanol to prevent bacterial contamination. Prior to loading the sample the column must be equilibrated with cold running buffer for at least 24 hours. If the column has been sitting more than a couple of months it may be necessary to empty the column, equilibrate the resin 3-4 washes in cold running buffer, and then re-pour the column. After the column is poured it should be equilibrated overnight so that the resin is completely packed.
10. Collect fractions of about 0.5 ml. Typically the DNA comes off between fractions 25 and 50. Load a 5 μl aliquot from a range of fractions to determine which fractions contain the backbone fragment. The small insert fragment will start to come off the column before all of the backbone is eluted, so it will be necessary to be conservative when fractions are pooled. For this reason typically 40-60% of the DNA is lost at this step.
11. Pool the fractions that contain the backbone.
12. Ethanol precipitate the samples. Resuspend in a volume that produces about 10-50 ng/μl.

13. Store at -70°C.

Column running buffer: (store at 4°C)

5 mM EDTA

100 mM NaCl

50 mM Tris-HCL pH 8.0

10 μg/ml tRNA (R-8759, Sigma)

I. Oligonucleotide Mutagenesis

Prepare Ampicillin-sensitive single stranded DNA of the template to be mutated. Design a mutagenic primer that will randomly generate all possible amino acid codons.

Mutagenesis reaction:

Table 11

Component	Final concentration
Single Stranded Template	0.05 pmol
Mutagenic Oligonucleotide	1.25 pmol
Ampicillin Repair Oligo (Promega q631a)	0.25 pmol
10X annealing buffer*	1X
Water to 20 μl	

*10X Annealing buffer:

-200 mM Tris-HCl, pH 7.5

-100 mM MgCl2

-500 mM NaCl

Heat reaction at 60°C for 15 minutes and then immediately place on ice.

Synthesis reaction:

Table 12

Component	Amount
Water	5 μl
10X synthesis buffer*	3 μl
T4 DNA Polymerase (Promega m421a)	1 μl (10 Units)
T4 DNA Ligase (Promega 180a)	1 μl (3 Units)

*10X Synthesis buffer

100 mM Tris-HCl, pH 7.5

5 mM dNTPs

10 mM ATP

20 mM DTT

Incubate at 37°C for 90 minutes.

Transform into Mut-S strain BMH 71-18 (Promega strain Q6321)

-Place Synthesis reaction in a 17X100 mm tube.

-Add BMH 71-18 competent cells that have been thawed on ice to synthesis reaction.

-Incubate on ice for 30 min

-Heat Shock cells at 42°C for 90 seconds.

-Add 4 ml of LB medium and grow cells at 37°C for 1 hour. Add Ampicillin to a final concentration of 1.25 ug/ml and then grow overnight at 37°C.

Isolate DNA with Wizard Plus Purification system (Promega a7100)

Transform isolated DNA into JM109 electrocompetent cells and transform onto LB Ampicillin plates.

J. Screening procedure

JM109 clones (from a transformation reaction) are plated onto nitrocellulose filters placed on LB amp plates at a screening density of about 500 colonies per plate.

As listed in the Random Mutagenesis procedure, approximately 10% of the clones to be selected will have to be as stable as the same sequenced or better than source. Or stated another way, about 50 colonies per plate will be suitable for selection. There are 704 wells available for a full eight plate robotic run, so at least 15 LB amp plates will be needed for a full robotic run.

After overnight growth at 37°C the plates containing the transformants are removed from the incubator and placed at room temperature.

The nitrocellulose filter is lifted on one side and 500 μl of 10 mM IPTG is added to each of the plates. The filter is then placed back onto the plate to allow diffusion of the IPTG into the colonies containing the different mutant luciferase genes. The plates are then incubated for about 4 hours at room temperature.

One (1) ml of a solution contains 1 mM luciferin and 100 mM sodium citrate is pipetted onto a slide warmer that is set at 50°C. A nitrocellulose filter that contains mutant luciferase colonies and has been treated with IPTG is then placed on top of the luciferin solution. After several minutes, the brightest colonies are picked with tooth picks which are used to inoculate wells in a microtiter plate that contain M9- minimal media with 1% gelatin.

After enough colonies are picked to 8 microtiter plates, the plates are placed in an incubator at 350 rpm at 30°C incubation and are grown overnight.

In the morning the overnight plates are loaded onto the robot and the cell dilution procedure is run. (This procedure dilutes the cultures 1:10 into induction medium). The new plates are grown for 3 hours at 350 rpm at 30°C.

After growth, the plates are loaded to the robot for the main assay procedure.

Minimal Media:

6 g/Liter Na_2HPO_4

3 g/Liter KH_2PO_4

0.5 g/Liter NaCl

1 g/Liter NH_4Cl

2 mM $MgSO_4$

0.1 mM

1 mM Thiamine-HCl

0.2% glucose

12 μg/ml tetracycline

100 μg/ml ampicillin

*Overnight media contains 1% gelatin

*Induction media contains 1 mM IPTG and no gelatin.

S.O.C. Media:

-10 mM NaCl

-2.5 mM KC1

-20 mM $MgC1_2$

-20 mM glucose

-2% bactotryptone

-0.5% yeast extract

Summary of Exemplary Evolutionary Progression

1. Start with Luc*Ppe2*[T249M]
2. Mutate 3 amino acids at N- and C-termini
3. Mutate 7 cysteines
4. Perform two iterations of evolution → Luc*49-7C6*
5. Mutagenesis of altered codons (9)
6. Two iterations of evolution → Luc*78-0B10*
7. Mutagenesis of consensus codons (28)
8. Mutagenesis of codon usage (24) → Luc*90-1B5*

One Iteration of Recursive Process

1. 1 clone → 3 libraries using error-prone PCR
   • 3 x Visual screen (about 10,000 clones each)
   • 3 x Quantitative screen (704) clones each)
2. 3 x 18 clones → library using sPCR
   • Visual screen (about 10,000 clones)
   • Quantitative screen (704 clones)
3. 18 + 18 → library using sPCR
   • Visual screen (about 10,000 clones)

- Quantitative screen (704 clones)

4. Output: 18 clones

EXAMPLE 5

Mutagenesis Strategy from Clone Luc90-1B5 to Luc133-1B2 and Luc146-1H2

Upon storage, luciferin degrades and the degradation products inhibit luciferase. The production of inhibitors causes an apparent instability in the reagent containing both luciferase and luciferin. There are two ways to reduce this problem: 1) Store the luciferin and luciferase at pH 5.5-6.0 to reduce the rate of luciferin degradation, and/or 2) Evolve an enzyme that is resistant to the luciferin degradation products.

Luc*Ppe2* mutants that were evolved after clone Luc90-1B5 were evolved to be more stable at low pH and have resistance to luciferin degradation products. These mutant enzymes are useful, for example, in an ATP detection kit. One embodiment of such a kit comprises a mixture of luciferin and luciferase. A luminescent reaction occurs when a sample comprising ATP is added to the mixture.

Three populations of random mutants were produced using clone Luc90-1B5 as a template. These three populations were screened on the robot as experiments 114, 115, and 117. Robotic screens for experiments 114, 115, 116, 117, 118,1 19, and 122 were completed as described previously except that buffer C was prepared with citrate buffer pH 4.5 instead of HEPES buffer pH 7.8, and the assay reagent was prepared with HEPES pH 7.1 with 10 μM ATP instead of Tricine pH 8.0 and 175 μM ATP. These screening conditions were biased to select clones that have increased retention of luminescence activity over time at pH 4.5 at 48°C and increased luminescence activity when assayed at pH 7.1 with 10 μM ATP. Seventeen clones from experiment 114, seven clones from experiment 115, and ten clones from experiment 116 were shuffled together using sPCR and selected mutants from this screen were run on the robot as experiment 117. Eighteen clones were selected from experiment 117.

The clone that was determined to have the most improved characteristics (increased retention of luminescence activity over time at pH 4.5 and 48°C and increased luminescence activity when assayed at pH 7.1 with 10 μM ATP) was clone Luc117-3C1 and it was selected as a template for random mutagenesis. Two populations of random mutants were screened and then run on the robot as experiments 118 and 119. Seven clones from experiment 118 and five clones from experiment 119 were saved.

Clones from experiments 114, 115, 116, 117, 118, and 119 were selected based upon the following characteristics: brighter luminescence than Luc90-1B5, and increased retention of luminescence activity over time at pH 4.5. These select clones were shuffled together and were run on the robot as experiment 122. Eleven clones from this experiment were saved.

Three populations of random mutants were prepared from clone Luc122-4D5 and run on the robot as experiments 125, 126, and 127. Thirteen clones from experiment 125, four clones form experiment 126, and three clones from experiment 127 were shuffled together and run on the robot as experiment 128. For experiments 125, 126, 127 and 128 the screen for K_m was altered to select for clones that are more resistant to luciferin degradation products. The clones were also screened for retention of luminescence over time at pH 4.5.

Instead of screening for substrate utilization, a screen for resistance to inhibitor was conducted. In place of the 0.06X dilution of substrates, a 75:25 mix of D to L luciferin in 1X assay buffer was used and designated as "0.75X". In place of the 0.02X dilution of substrates, a 50:50 mix of D to L luciferin in 1X assay buffer and was designated as "0.5X". The 1X assay buffer in these experiments contained the following: 10 μM ATP, 50 mM HEPES pH 7.8, 8 mM $MgSO_4$, and 0.1 mM EDTA. The 0.75X sample contained 75 μM D-luciferin and 25 μM L-luciferin. The 0.5X sample contained 50 μM D-luciferin and 50 μM L-luciferin. The 1X sample contained 250 μM D-luciferin. A K_m regression was used as before and a K_m value was calculated. Normalized values of greater than 1 indicate more resistance to inhibitor. Clones from these experiments that were shown to have greater resistance to L-luciferin were also more resistant to luciferin degradation products.

To more easily measure resistance to inhibitor on the robotic system, a new variable "Q" was designated. The "Q" variable replaces the K_m variable used previously. The luminescence ratio is calculated the same as in the K_m measurement, then the natural log (ln) of each luminescence ratio is calculated (Y-axis). The X-axis is an arbitrary time that is entered by the user. The first time point is zero and the samples are measured with 1X assay buffer that contains

250 µM D-luciferin. The next two time points have the same time value (i.e., 4 hours to simulate incubation of luciferin) and samples are measured with 1X assay buffer that contains a 50:50 mixture (as described above) of D-luciferin to L-luciferin. A linear regression correlating ln(lum ratio) to time is calculated. Q is calculated as the ln(0.5)/slope. Normalized values of "Q" greater than 1 indicate more resistance to inhibitor. Experiments 133 and higher were run using this program.

Sixteen clones from experiment 128 were shuffled with clones from experiment 122 and run on the robot as experiment 133. Two samples, Luc133-1B2 and Luc133-0D11, were selected as templates for random mutagenesis and run on the robot as experiments 145 and 146, respectively. The clone that showed an increased retention of luminescence over time at pH 4.5 and the most resistance to inhibitor was clone Luc146-1H2. Moreover, at pH 4.5 and 48°C, Luc133-1B2 and Luc146-1H2 had increased thermostability relative to Luc90-1B5, and increased resistance to inhibitor (Figures 54-61). A comparison of the luminescence signal for Luc49-7C6, Luc78-0B10, Luc90-1B5, Luc133-1B2, and Luc146-1H2 is shown in Figure 59. A comparison of the thermostability at 50°C for clones for Luc49-7C6, Luc78-0B10, Luc90-1B5, Luc133-1B2, and Luc146-1H2 is shown in Figure 60. Figures 55-58 show the nucleotide sequence encoding and the inferred amino acid sequence of Luc133-1B2 and Luc146-1H2.

<u>Materials and Methods</u>

<u>Assay to detect resistance to luciferase inhibitor</u>

A 10 mM stock solution of luciferin is incubated at 50°C in 50 mM HEPES, pH 7.8, to accelerate the production of luciferin breakdown products. At different time points an aliquot is removed and then placed at –20°C. After incubation is complete, assay reagent (100 µM Luciferin, 1 µM ATP, 50 mM

HEPES, pH 7.8 and 8 mM $MgSO_4$) is prepared with luciferin from each of the different time points and a diluted lysate is then assayed with each assay reagent.

The lysate is prepared as follows. Overnight cultures of clones to be tested are prepared in LB supplemented with 100 μg/ml AMP. The cultures are diluted 1:10 in M-9 minimal media supplemented with 1 mM IPTG, 100 μg/ml AMP and grown for 3 hours at 30°C. Forty-five μl of cells is mixed with 20 μl of Buffer A and frozen. The mixture is thawed, 175 μl of Buffer B added, and the resulting mixture diluted 1:10 in Buffer C. A regression of luminescence versus time of luciferin incubation is then calculated, and from this graph half-life is extrapolated. A longer half-life means that the mutant being tested is more resistant to luciferin breakdown products.

EXAMPLE 6

Mutagenesis Strategy from LucPplYG to Clone Luc81-6G01

The luciferases from the luminous beetle, *Pyrophorus plagiophthalamus,* had been shown previously to generate different colors of luminescence (Luc*Ppl*). Analysis of these luciferases revealed that the different colors were caused by discreet amino acid substitutions to their protein sequences. This allowed the possibility to make a pair of genetic reporters capable of emitting a multiplexed luminescent signal, thus enabling quantitation of two biomolecular events simultaneously from within the same living system.

Amino acid substituted Luc*Ppl* were prepared which have the following properties:

Physical stability of the luciferases

Although the luminescence activity of Luc*Ppl* within colonies of *E. coli* appeared to be thermostable to above 60°C, in lysates these luciferases had relatively low stability. They were particularly unstable in the presence of Triton X-100 detergent. When lysates are prepared containing the commonly used firefly luciferase, the enzyme retains greater than 90% activity over 5 hours at room temperature. In contrast, the activity of the Luc*Ppl* luciferases would decrease several fold over the same period.

The thermostabilities of the Luc*Ppl* luciferases are also near the physiological temperature of mammalian cells. The green-emitting luciferase

(Luc*Ppl*GR) and red-emitting luciferase (Luc*Ppl*RD) have different thermostabilities which may cause differences in the behaviors as genetic reporters within cells. The influence of temperature should be greatest near the point of denaturation for the enzymes, where small changes in temperature will have the greatest effect on protein structure. In contrast, temperature will have much less affect on protein structure when it is much below the denaturation point. Thus, the differential effect on two enzymes having slightly different denaturation temperatures will be less at relatively lower temperatures. It might therefore be preferable to have the denaturation temperature of the reporter enzyme significantly above the growth temperature of mammalian cells.

Spectral overlap between the luciferases

Although a method was developed to quantify each luciferase in a mixture by using colored filters, the ability to discriminate between the luciferases is limited by their spectral overlap. This overlap reduces the ability to accurately measure both luciferases if their luminescence intensities differ by more than 10 fold. If the intensities differ by more than 50 fold, the luminescence signal of the dimmer luciferase is obscured by the other. Thus, it would be preferable to further separate the luminescence spectra of the two luciferases.

Many different mutations were identified which shifted the luminescence spectrum towards the red. But the limit for red luminescence appears to be about 620 nm. Further effort at shifting the spectrum of the red-emitting luciferase into still longer wavelengths might have some benefit. It was found that green-shifting mutations were rare, however, an extensive analysis was not conducted. Measurements from native luciferases show some examples of luminescence below 530 nm, about 15 nm less than the green-emitting prototype enzyme.

Differential physical and enzymological characteristics

Ideally the two luciferase reporters would be identical in all characteristics except for the color of luminescence. However, as noted above, the physical stability of the luciferases was not identical. It was also found that mutations resulting in red-shifted luminescence also caused an increase in the K_M for luciferin. Although some of these differences may be unavoidable, it is not clear whether the properties are fundamentally associated. For instance,

luciferases from different beetle species sometimes have significantly differing K_M even though their luminescence spectra are similar. It may be that much of the differences associated with development of the red-emitting luciferase are due to concomitant perturbations to the integrity of the enzyme structure, as the thermostability of a prototype of the red-emitting luciferase was increased without significantly altering the luminescence spectrum.

Stable luminescence signals

When firefly luciferase was first described as a genetic reporter, the luminescent signal was a relatively brief flash initiated upon injection of the reaction substrates. Subsequent development of the luminescent chemistry made the assay more convenient by enabling a stable signal for several minutes. Presently, such stabilized assays are standard for general laboratory applications. However, to allow high throughput screening in pharmaceutical research, the luminescence signal was further stabilized to extend for over an hour. This was necessary to allow sufficient time to assay several thousand samples in a batch. Although the luminescent signal of the new multiplexed luciferases was stable for minutes, they did not provide the extended signal stability needed for high throughput screening. It would be preferable if the signal stability could be further increased while optimizing other properties.

Methods to Optimize Luciferase Performance

To prepare luciferases having certain performance, a method for *in vitro* evolution of enzyme function, as described above, was employed. Briefly described, the method is a recursive process of generating random mutations and screening for desirable properties. It was originally developed primarily to increase the thermostability of luciferases, although other enzymological characteristics are also subject to optimization by the screening criteria. A slightly different strategy was used to achieve the properties described above since two related luciferases needed to be optimized concomitantly.

Initially, a single prototype enzyme is subjected to *in vitro* evolution to optimize physical stability and the luminescence signal. In the process, the mutant libraries are also screened for any new mutations causing changes in color. Particular emphasis is placed on isolating green shifted mutants. After initial optimization of a common prototype, a green- and red-emitting form of

the enzymes is created, and these are further optimized separately to harmonize their physical and chemical properties. Particular attention is given to matching their physical stabilities and their substrate binding constants, especially for luciferin.

The choice for the initial prototype for optimization was the wild-type yellow-green-emitting luciferase isolated from the luminous beetles (Luc*Ppl*YG). Of the luciferases originally cloned from *P. plagiophthalamus*, this one produced the brightest luminescence when expressed in *E. coli*. Furthermore, there was concern that the lack of green-shifting mutation resulted because the prior mutagenesis studies were done using a luciferase that already had the greenest luminescence. It was possible that if additional green-shifting mutations existed, they might be more evident when screened in a red-shifted background. The mutagenesis was performed as follows:

Remove peroxisomal targeting sequence

The translocation signal at the C-terminus of the luciferases was removed. This was done using oligonucleotide-directed mutagenesis to convert the normal –KSKL to –XXX* (where X represents any amino acid, and * represents a termination codon). Several colonies yielding bright luminescence were selected and used as templates for the next stage of mutagenesis.

Removal of sensitive cysteines

The luciferases from *P. plagiophthalamus* have 13 cysteines, which are potentially sensitive to oxidation. This is in contrast to the commonly used firefly luciferase, which has only 4 cysteines. To remove any cysteines that may limit enzyme stability, oligonucleotide-directed mutagenesis was used to randomize the cysteine codons. Three sets of oligonucleotides were used: non-conserved cysteines in regions of low sequence homology (positions 69, 114, 160, 194, 335, and 460), non-conserved cysteines in regions of higher sequence homology (positions 127, 213, 310, and 311), and highly conserved cysteines (positions 60, 80, and 388). The best clones from each of these screens were isolated, and a new mutant library made by sPCR and screened again. At the screening temperature of 29°C, the activity of the wild-type yellow-green-emitting luciferase decreased about 500-fold over 10 hours. The activity of the most stable mutant (Luc20-4C10) was more stable, decreasing only about 2 fold.

First cycle of random mutagenesis

Using the procedure developed previously, three mutant libraries were generated using error-prone PCR and screened. The best mutants from these were recombined into a new library by sPCR and screened again. Finally, the best clones of this screen were recombined with the best clones from the previous oligonucleotide-directed mutagenesis by sPCR, and screened again. At 41°C, the activity of the best mutant from this process (Luc30-4B02) decreased 63 fold over 10 hours, whereas the activity of the parent mutant (Luc20-4C10) decreased greater than 100,000 fold.

Sequence analysis

Six of the best mutants from the last screen were isolated and sequenced. This revealed that the amino acids at 16 positions had been changed among the six clones. Thirteen positions had been changed in the preferred mutant, Luc30-4B02. Four of the changes were at the C-terminus in all the isolated mutants, where oligonucleotide mutagenesis had changed the wild-type sequence of –KSKL to –AGG*. Only two of the cysteines had been changed by the previous oligonucleotide mutagenesis; one highly conserved cysteine at position 60 was changed to valine and one moderately conserved cysteine at position 127 was changed to threonine. The remaining amino acid changes were all due to point mutations in the DNA, consistent with error-prone PCR. Interestingly, three of these changed the amino acid into that found in the wild-type green-emitting luciferase (two in mutant Luc30-4B02). Four of the remaining changes brought the mutant sequences closer to the consensus amino acid among other cloned beetle luciferases (two in mutant Luc30-4B02) (Figure 19B). An additional 4 codons were changed without affecting the amino acid sequence.

Site-directed mutagenesis

To further explore the potential of the mutations identified in the sequenced mutants, additional mutagenesis experiments were performed using oligonucleotides. Eight of the codons mutated by the error-prone PCR were randomized or partially randomized using oligonucleotide-directed mutagenesis. Four of the remaining cysteine codons were randomized; two highly conserved cysteines (positions 80 and 388) and two cysteines in a region of sequence homology (positions 310 and 311). One leucine was mutated to a

leucine/proline; proline is the consensus amino acid among other beetle luciferases.

The mutagenesis was performed with four sets of oligonucleotides (Table 13), and the best clones from each set were selected. These were recombined by sPCR together with the selected clones from the previous random mutagenesis and screened again. The activity of the best clone from this process (Luc47-7A11) decreased 2.3 fold at 42°C; the activity of the parent clone (Luc30-4B02) decreased greater than 2000 fold.

Table 13

Experiment	Mutations
Set A	C_{80}→X + K_{84}→X + L_{91}→(F, L, I, M, V, S, P, T, A)
Set B	I_{288}→(F, L, I, M, V, S, P, T, A) C_{310}→X + C_{311}→X
Set C	G_{351}→(I, M, V, T, A, N, K, D, E, S, R, G) + L_{350}→(L, P) + S_{356}→P + L_{359}→(F, L, I, M, V, S, P, T, A)
Set D	C_{388}→X + V_{389}→(NYN) K_{457}→X

Second cycle of random mutagenesis

The random mutagenesis process using error-prone PCR was applied again to the best clone from the oligonucleotide-directed mutagenesis (Luc47-7A11). Three libraries were again created and screened, and selected mutants were recombined by sPCR and screened again. Following recombination, the activity of the best mutant (Luc53-0G01) decreased 1.2 fold at 43°C. The parent clone (Luc47-7A11) decreased 150 fold. After recombining the best of these new mutants with the best mutants from the previous oligonucleotide-directed mutagenesis, the activity of the new best mutant (Luc55-2E09) decreased 31 fold at 47°C, compared to 80 fold for the parent (Luc53-0G01).

Third cycle of random mutagenesis

The random mutagenesis process was repeated using the best clone from the previous cycle of mutagenesis (Luc53-0G01). After recombining the selected mutants with the mutants from the second cycle of mutagenesis, the activity of the best clone (Luc81-6G01) decreased 100-fold at 47°C, compared with 750 fold for the parent (Luc53-0G01). The discrepancy in measured activity of Luc53-0G01 in this cycle of mutagenesis compared to the previous

cycle may be due to changes in the assay procedure and recalibration of the incubator temperature. It should be noted that the recorded thermostabilities from each stage of mutagenesis are calculated from robotic data using abbreviated assay procedures. The data are intended to indicate the relative stabilities of enzyme mutants when assayed in the same screen, rather than providing an accurate quantitation of thermostability.

Luminescence

Before making a final selection of the best clone from which to create the green- and red-emitting luciferases, further analysis was done on the best clones from the final screen. Three clones in particular were strong candidates as the final choice: Luc81-0B11, Luc81-5F01, and Luc81-6G01. The luminescence properties of these three mutant enzymes were compared among one another. They were also compared to the wild-type yellow-green-emitting luciferase to gauge the effect of the *in vitro* evolution process.

From colonies of *E. coli* expressing the luciferases, Luc81-5F01 and Luc81-6G01 produced luminescence most rapidly at room temperature upon addition of luciferin. The luminescence was more rapid and brighter than colonies expressing the wild-type green- and yellow-green-emitting luciferases. The luminescence from all the selected colonies appeared green-shifted compared to the yellow-green parent clone. When the colonies are heated to 65°C, the yellow-green clone looses most luminescence and the green clone becomes dimmer. Some of the mutant clones loose their luminescence at 65°C, but the three preferred clones remain bright above 70°C. No spectral changes upon heating the colonies were evident until above 70°C, where those clones still retaining activity began to red-shift slightly (sometime, the initial phases of enzyme denaturation are accompanied by a red shift in the luminescence). The luminescence characteristics of the three preferred mutants are quite similar.

The thermostability of the mutant luciferases in cell lysates was compared at room temperature (Figure 63). Dilute lysates were buffered at pH 7.5 and contained 1% Triton X-100; typical conditions for lysates of mammalian cells. The luminescence activity of all three mutant enzyme showed no decrease

over 20 hours, whereas the activity of the wild-type yellow-green-emitting luciferase decreased substantially.

For luminescence assays requiring only a few second, the wild-type yellow-green-emitting luciferase produces a very stable signal (the initial rise in the signal evident in the first 2 seconds is due to the response time of the luminometer, not the kinetics of the luminescence reaction) (Figure 64A). However, the signal intensity was reduced about 30% by the presence of 1% Triton X-100 in the lysate (diluted 1:5 with the addition of assay reagent). In contrast, the luminescence intensity of the mutant luciferases was unaffected by the presence of Triton X-100. Under these conditions, the most stable signal was produced by Luc81-6G01, although the signal intensity was somewhat brighter for Luc81-5F01. However, the data are not corrected for the efficiency of enzyme expression in *E. coli*. Thus, differences in luminescence intensity may not correlate to changes in enzyme specific activity, nor is the expression efficiency in *E. coli* necessarily relevant to expression in mammalian cells.

For batches of assays requiring more than an hour to process, the signal stability of the yellow-green-emitting luciferase is inadequate under the conditions tested. The luminescence intensity decreases several fold per hour (Figure 64B). Attempts to correct this by the *in vitro* evolution yielded mixed results. The signal stability of all three mutant enzymes was generally much improved over the parent yellow-green enzyme for three hours after substrate addition. However, this was accompanied by a greater initial decrease in luminescence during the first half-hour. This initial decrease would be more acceptable if it had occurred more rapidly, so that batch processing of samples would not be delayed by 30 minutes in waiting for the signal to stabilize. It may be possible to improve this kinetic behavior by adjusting the assay conditions.

From these results, the mutant Luc81-6G01 was chosen as the best clone from which to subsequently create the green- and red-emitting luciferases. The sequence of Luc81-6G01 (Figures 46-47) and Luc81-0B11 was determined and compared with the sequences of Luc30-4B02 from earlier in the *in vitro* evolution process, and the wild-type yellow-green-emitting luciferase used as the initial parent clone (Figure 19B). Relative to Luc30-4B02, the Luc81-6G01 mutant acquired new mutations in 9 codons, of which 8 caused changes in the

amino acid sequence. Four of these 8 amino acid changes were probably acquired through recombination with clones generated prior to isolation of Luc30-4B02. Two are identical to mutations found in the other clones sequenced along with Luc30-4B02, and two are reversions to the wild-type parent sequence. The remaining four are novel in the sequence of Luc81-6G01. Two of the novel mutations change the amino acid to the consensus amino acid among other cloned luciferases.

Interestingly, in either the sequences of Luc81-6G01 or Luc81-0B11, there is no evidence that the prior oligonucleotide-directed mutagenesis had any beneficial effect. No novel nucleotide sequences appear at any of the targeted codons. The improved enzyme performance following the oligonucleotide-directed mutagenesis apparently was due to recombination of previously acquired mutations. All of the novel amino acid changes in Luc81-6G01 and Luc81-0B11 are at sites not targeted by the oligonucleotides and are due to single-base modifications of the codons, consistent with error-prone PCR. Even though the novel mutations in Luc81-6G01 were not found in the earlier sequence data, it is not certain when they were generated in the process. Most likely they were produced in the second and third cycles of random mutagenesis; however, they may have been present among other selected mutants prior to Luc30-4B02. Relative to the initial yellow-green-emitting luciferase, the Luc81-6G01 mutant has acquired 17 amino acid changes and 3 codon mutations not affecting the amino acid sequence.

The observation that the onset of luminescence within colonies of *E. coli* is faster for the new mutants, and that the luminescence is brighter at higher temperatures, is probably not due to differences in protein expression. Immunoblot analysis of cell expressing the different luciferases showed no significant differences in the amount of polypeptide present. As noted above, the greater light intensity at higher temperatures is due to the increased thermostability of the mutant luciferases. The apparent K_M's for ATP and luciferin have also changed during the course of the *in vitro* evolution (Table 14). To estimate the K_M values, the mutant luciferases were partially purified from lysates of *E. coli* by differential precipitation using ammonium sulfate (40-

65% saturation fraction). The results show that the K_M's for both ATP and luciferase are more than 10-fold lower.

When luciferin is added to an *E. coli* colony expressing luciferase, the intracellular concentration of luciferin slowly increases as it diffuses across the cell membrane. Thus, the intracellular concentration of luciferin reaches saturation sooner for those luciferases having the lowest K_M's. Hence, the mutant luciferases appear brighter sooner than the wild-type parent clone. This also explains why the luminescence of the red-emitting prototype clone appears in *E. coli* colonies much more slowly than the green-emitting luciferase. Analysis of K_M shows that the mutations causing the red luminescence also substantially increase the K_M for luciferin.

Table 14

Luciferase	K_M for ATP (μM)	K_M for luciferin (μM)
YG w.t.	140	21
Luc30-4B02	12	7.8
Luc81-6G01	8.0	1.9

From the analysis of luminescence signal *in vitro*, the luminescence from the mutant luciferases might be expected to fade more quickly than the wild-type luciferase during the first 30 minutes. Following this, the luminescence should be most stable in the mutants. However, this has not been noticed in the colonies of *E. coli*, and it may be that the kinetics of luminescence are different within cells compared to diluted enzyme in buffer.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A second luciferase that has enhanced resistance to an inhibitor of the luciferase relative to a first reference beetle luciferase.

2. The second luciferase of claim 1 which retains at least 50% more activity in the presence of the inhibitor relative to the reference beetle luciferase.

3. The second luciferase of claim 1 which retains at least 100% more activity in the presence of the inhibitor relative to the reference beetle luciferase.

4. The second luciferase of claim 1 which comprises a plurality of amino acid substitutions relative to the reference beetle luciferase.

5. The second luciferase of claim 4 wherein the reference luciferase is native beetle luciferase.

6. The second luciferase of claim 5 wherein the reference beetle luciferase is Luc*Ppl*.

7. The second luciferase of claim 5 wherein the reference beetle luciferase is Luc*Ppe2*.

8. The second luciferase of claim 4 wherein the substitutions are to a consensus amino acid.

9. A luciferase which comprises SEQ ID NO:14, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:47, or an enzymologically active portion thereof.

10. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the luciferase of claim 9 or the complement thereof.

11. The isolated and purified nucleic acid molecule of claim 10 comprising a nucleic acid segment comprising SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:42, SEQ ID NO:43 or SEQ ID NO:46.

12. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the second luciferase of claim 1 or the complement thereof.

13. A vector containing the nucleic acid molecule of claim 10.

14. A vector containing the nucleic acid molecule of claim 12.

15. A host cell, the genome of which is augmented with the nucleic acid molecule of claim 10.

16. A host cell, the genome of which is augmented with the nucleic acid molecule of claim 12.

17. A solid substrate comprising the second luciferase of claim 1.

18. A solid substrate comprising the luciferase of claim 9.

19. A fusion protein comprising the second luciferase of claim 1.

20. A fusion protein comprising the luciferase of claim 9.

21. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the fusion protein of claim 19.

22. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the fusion protein of claim 20.

23. A method of using a luciferase, comprising: linking an agent with the second luciferase of claim 1 so as to yield a labeled agent.

24. The use of the second luciferase of claim 1 for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce hybrid proteins, for high temperature reactions, or for creating luminescent solutions.

25. A method of using a luciferase, comprising: linking an agent with the luciferase of claim 9 so as to yield a labeled agent.

26. The use of the luciferase of claim 9 for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce hybrid proteins, for high temperature reactions, or for creating luminescent solutions.

27. A method of using a vector encoding a luciferase, comprising:
a) introducing the vector of claim 13 into a host cell; and
b) detecting or determining the presence of luciferase in the host cell.

28. A method of using a vector encoding a luciferase, comprising:
a) introducing the vector of claim 14 into a host cell; and
b) detecting or determining the presence of luciferase in the host cell.

29. A kit comprising: a container comprising the second luciferase of claim 1.

30. The kit of claim 29 wherein the container comprises an aqueous mixture comprising the luciferase.

31. The kit of claim 29 wherein the container comprises lyophilized luciferase.

32. The kit of claim 29 further comprising a container comprising luciferin.

33. The kit of claim 29 wherein the container further comprises luciferin.

34. The kit of claim 31 wherein the container further comprises lyophilized luciferin.

35. The kit of claim 34 wherein the container which comprises luciferin comprises lyophilized luciferin.

36. The kit of claim 29 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having ATP with the level of ATP in the sample.

37. The kit of claim 29 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having an infectious agent that produces ATP with the level or presence of the agent in the sample.

38. A kit comprising: a container comprising the luciferase of claim 9.

39. The kit of claim 38 wherein the container comprises an aqueous mixture comprising the luciferase.

40. The kit of claim 38 wherein the container comprises lyophilized luciferase.

41. The kit of claim 38 further comprising a container comprising luciferin.

42. The kit of claim 38 wherein the container further comprises luciferin.

43. The kit of claim 40 wherein the container further comprises lyophilized luciferin.

44. The kit of claim 41 wherein the container which comprises luciferin comprises lyophilized luciferin.

45. The kit of claim 38 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having ATP with the level of ATP in the sample.

46. The kit of claim 38 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having an infectious agent that produces ATP with the level or presence of the agent in the sample.

47. A method to prepare an enzyme which is not a beetle luciferase and which has enhanced enzymological properties, comprising:
a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is not a luciferase and which has at least one enhanced enzymological property from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme which is not a luciferase, wherein the first isolated polynucleotide sequence is subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has at least one enhanced enzymological property relative to the enzyme encoded by the first isolated polynucleotide sequence;
b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences, wherein the selected isolated polynucleotide sequence is subjected to oligonucleotide mediated mutagenesis with a plurality of oligonucleotides each

comprising at least one codon that encodes a consensus amino acid which is not present in the first polynucleotide sequence; and

c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme which is not a luciferase having at least one enhanced enzymological property and comprising a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence.

48. The method of claim 47 further comprising isolating the further polynucleotide sequence.

49. The method of claim 47 wherein in step b) a mixture of the selected isolated polynucleotide sequences of step a) are mutated.

50. The method of claim 47 wherein the property is specific activity, enzyme activity, catalytic turnover, Km or substrate utilization.

51. The method of claim 47 wherein the enzyme is DNA polymerase or RNA polymerase.

52. The method of claim 47 where the enzyme is detectable in crude cellular lysates or cells.

53. The method of claim 52 wherein the enzyme is chloramphenicol acetyltransferase, beta-glucuronidase or beta-galactosidase.

54. The method of claim 47 wherein the first polynucleotide sequence is subjected to recombination mutagenesis.

55. The method of claim 47 wherein the first polynucleotide sequence is subjected to point mutagenesis.

56. The method of claim 47 wherein the selection is an automated multi-parameter process.

57. A polynucleotide sequence which is obtained by the method of claim 47.

58. A method to prepare an enzyme that is resistant to an inhibitor, comprising:
a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the enzyme encoded by the first isolated polynucleotide sequence;
b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and
c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence.

59. The method of claim 58 further comprising isolating the further polynucleotide sequence.

60. The method of claim 58 wherein in step b) a mixture of the selected isolated polynucleotide sequences of step a) are mutated.

61. The method of claim 58 wherein the further polynucleotide sequence encodes an enzyme that has increased thermostability relative to the first polynucleotide sequence.

62. The method of claim 58 wherein the enzyme is DNA polymerase or RNA polymerase.

63. The method of claim 58 wherein the enzyme is resistant to inhibition by a substrate analog of the enzyme.

64. The method of claim 58 wherein the mutating employs oligonucleotides having at least one codon encoding a consensus amino acid.

65. The method of claim 58 wherein the enzyme is a luciferase.

66. The method of claim 65 wherein the luciferase is a beetle luciferase.

67. The method of claim 66 wherein the first polynucleotide sequence encodes Luc*Ppe2*.

68. The method of claim 66 wherein the first polynucleotide sequence encodes Luc*Ppl*.

69. The method of claim 58 wherein a plurality of amino acid substitutions are to a consensus amino acid.

70. The method of claim 58 wherein the first polynucleotide sequence is subjected to recombination mutagenesis.

71. The method of claim 58 wherein the first polynucleotide sequence is subjected to point mutagenesis.

72. The method of claim 58 wherein the selection is an automated multi-parameter process.

73. The method of claim 65 wherein the luciferase has increased luminescence intensity, increased signal stability or decreased Km.

74. A polynucleotide sequence which is obtained by the method of claim 58.

75. An enzyme which is encoded by the polynucleotide sequence of claim 57.

76. An enzyme which is encoded by the polynucleotide sequence of claim 76.

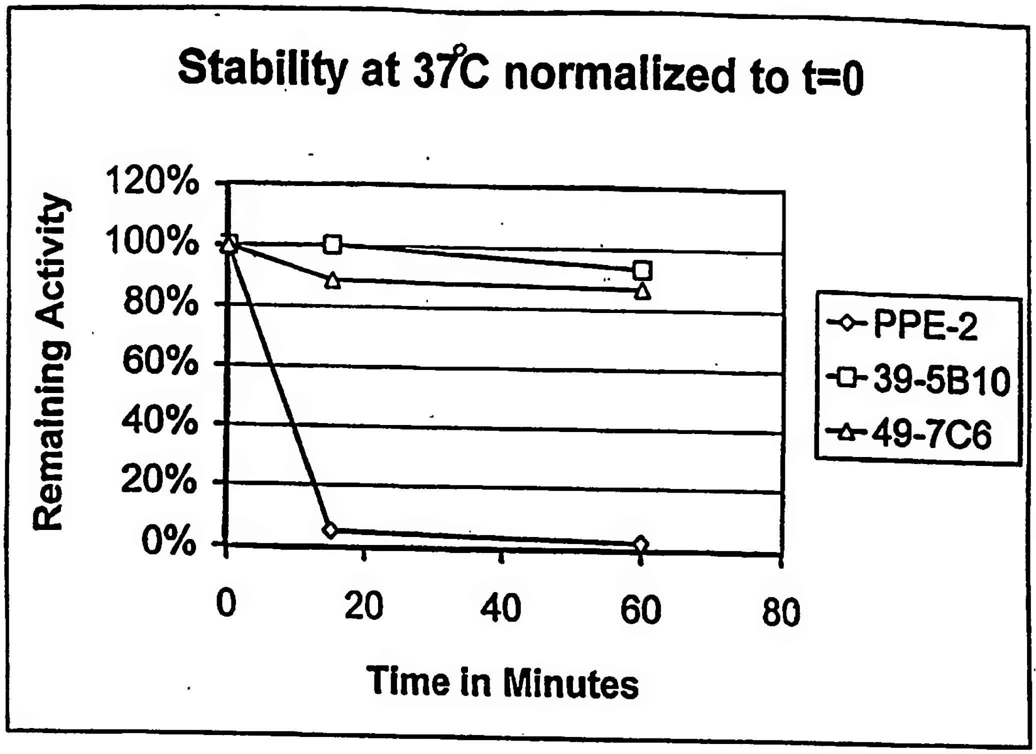

Stability at 37°C normalized to t=0
Remaining Activity
120%
100%
80%
60%
40%
20%
0%
0
20
40
60
80
Time in Minutes
PPE-2
39-5B10
49-7C6

FIG. 1

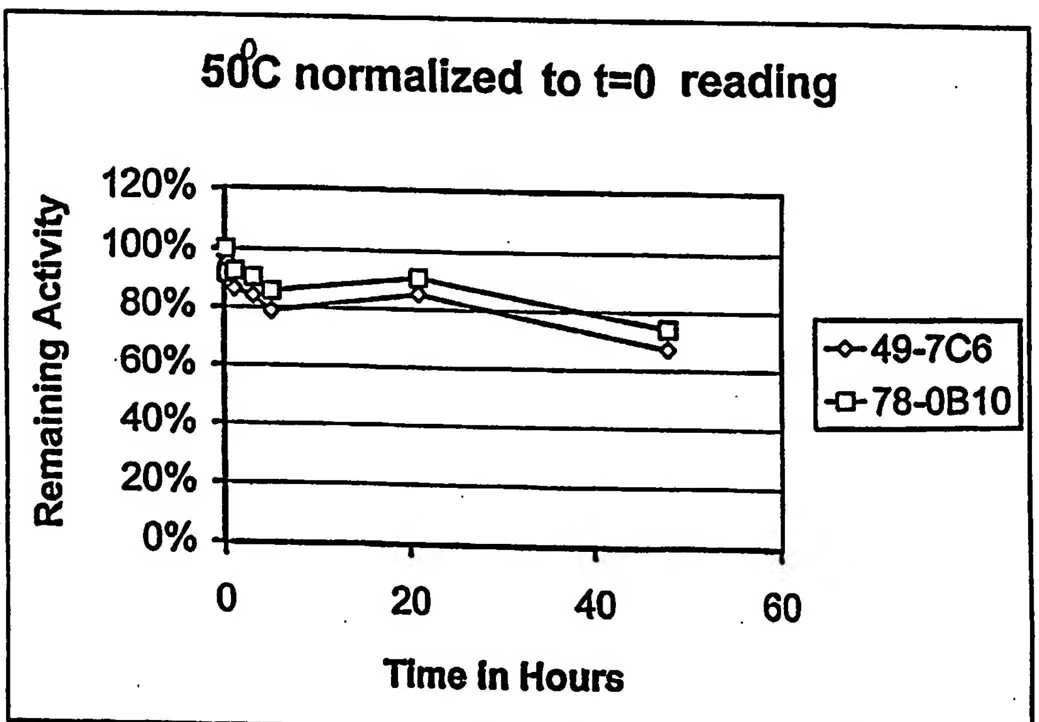

50°C normalized to t=0 reading
Remaining Activity
120%
100%
80%
60%
40%
20%
0%
0
20
40
60
Time in Hours
49-7C6
78-0B10

FIG. 2

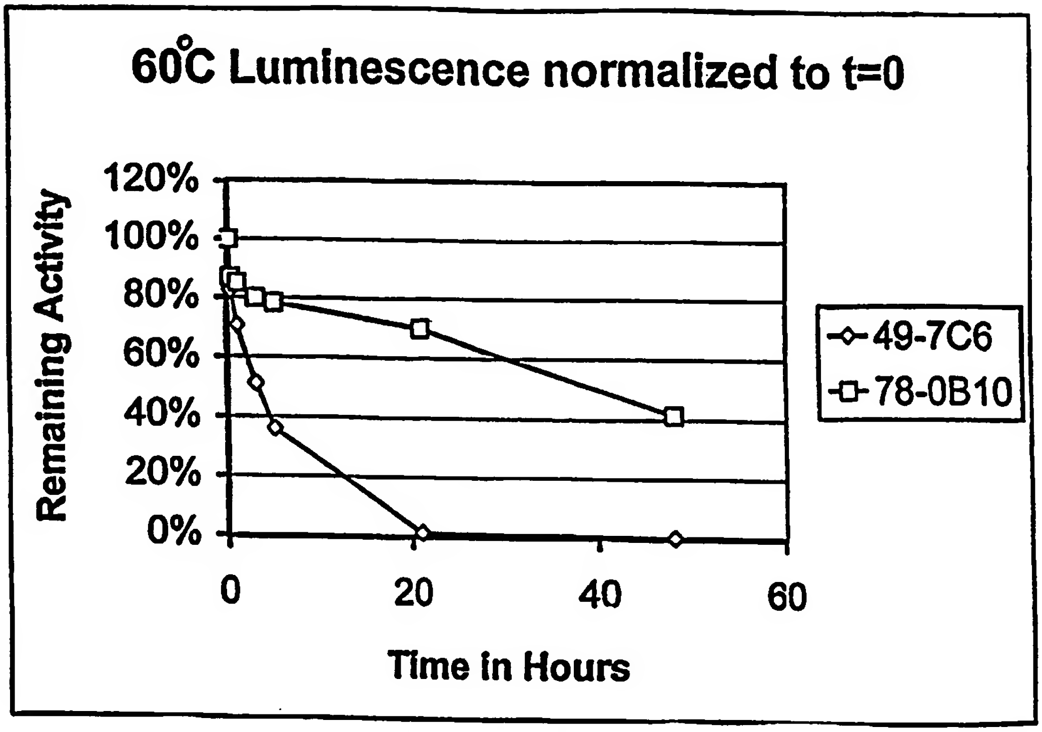

60°C Luminescence normalized to t=0
Remaining Activity
120%
100%
80%
60%
40%
20%
0%
0
20
40
60
Time in Hours
49-7C6
78-0B10

FIG. 3

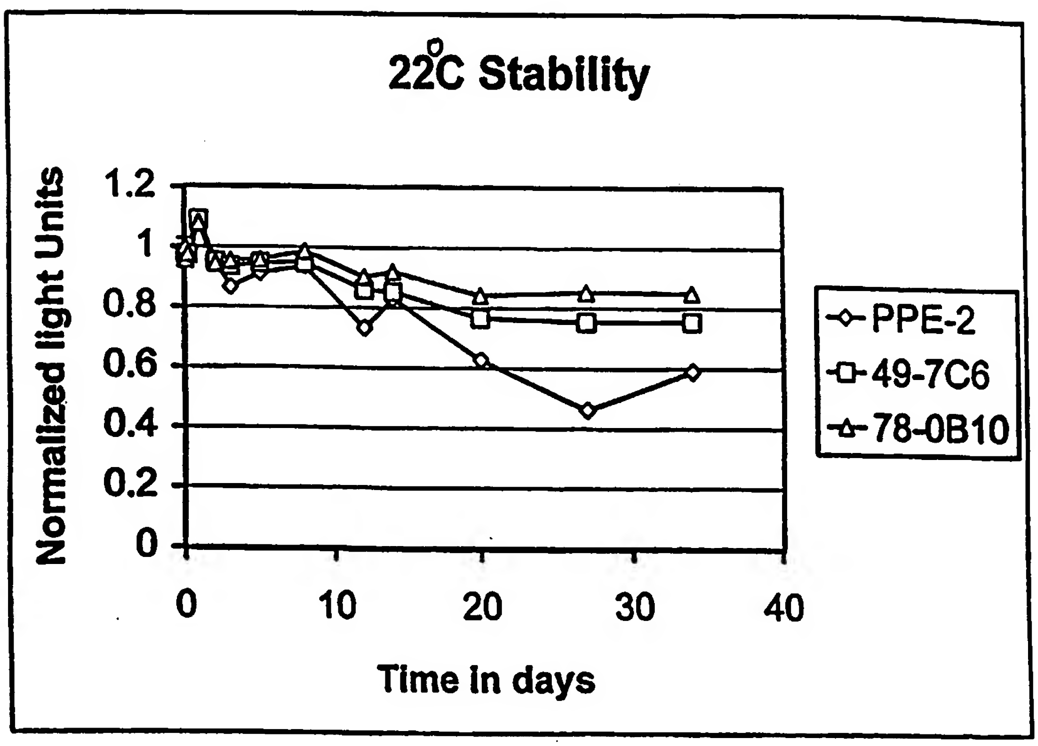

22°C Stability
Normalized light Units
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
Time in days
PPE-2
49-7C6
78-0B10

FIG. 4

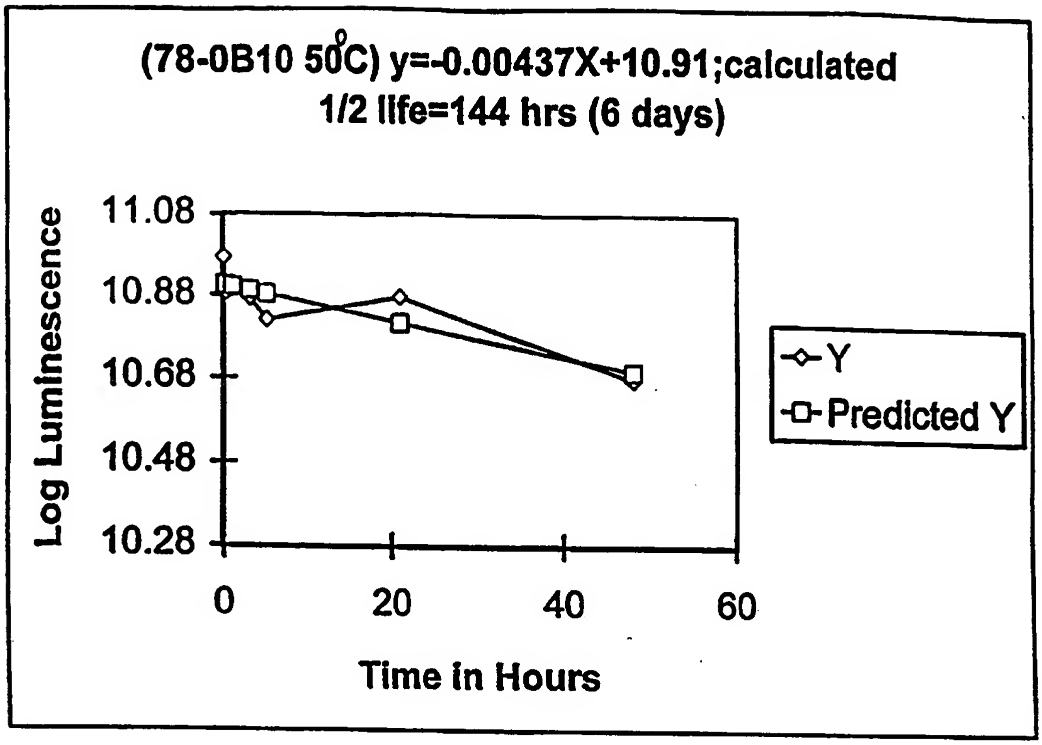

(78-0B10 50°C) y=-0.00437X+10.91;calculated 1/2 life=144 hrs (6 days)
Log Luminescence
11.08
10.88
10.68
10.48
10.28
0
20
40
60
Time in Hours
Y
Predicted Y

FIG. 5

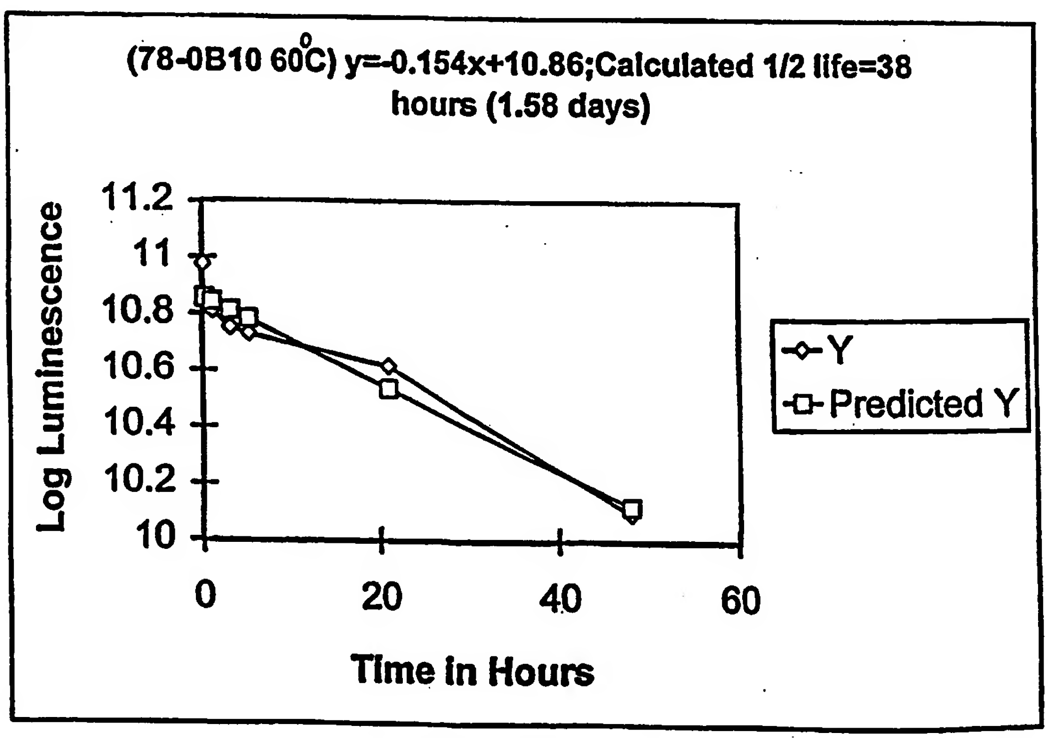

(78-0B10 60°C) y=-0.154x+10.86;Calculated 1/2 life=38 hours (1.58 days)
Log Luminescence
11.2
11
10.8
10.6
10.4
10.2
10
0
20
40
60
Time in Hours
Y
Predicted Y

FIG. 6

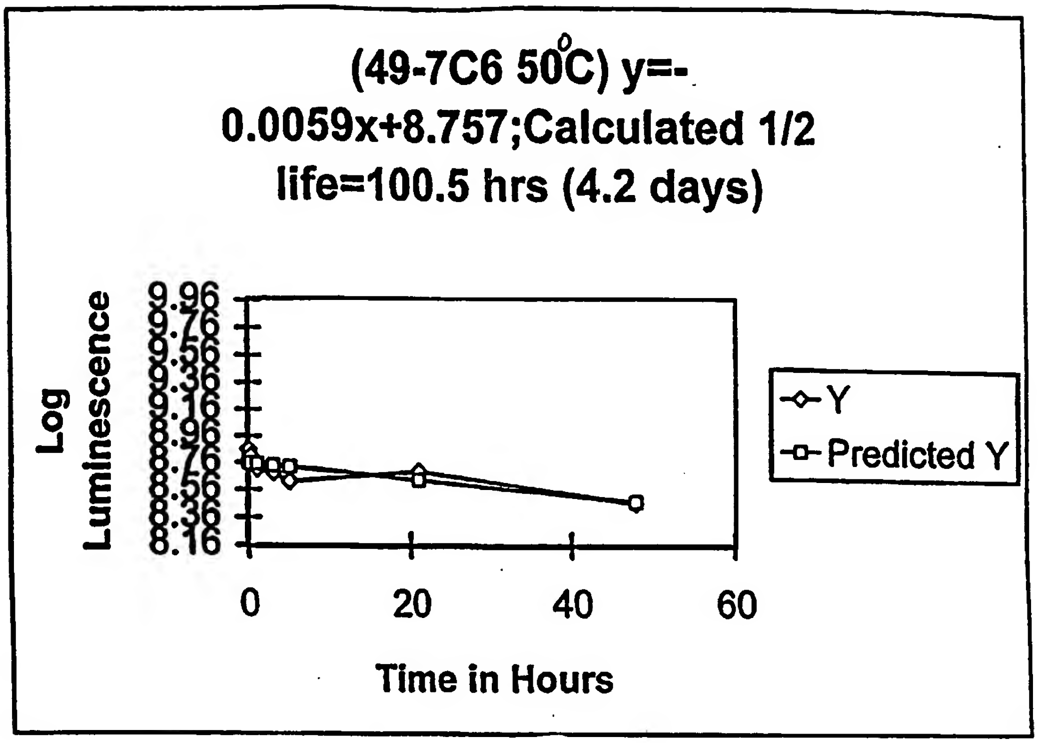

(49-7C6 50°C) y=-0.0059x+8.757;Calculated 1/2 life=100.5 hrs (4.2 days)
Log Luminescence
9.96
9.76
9.56
9.36
9.16
8.96
8.76
8.56
8.36
8.16
0
20
40
60
Time in Hours
Y
Predicted Y

FIG. 7

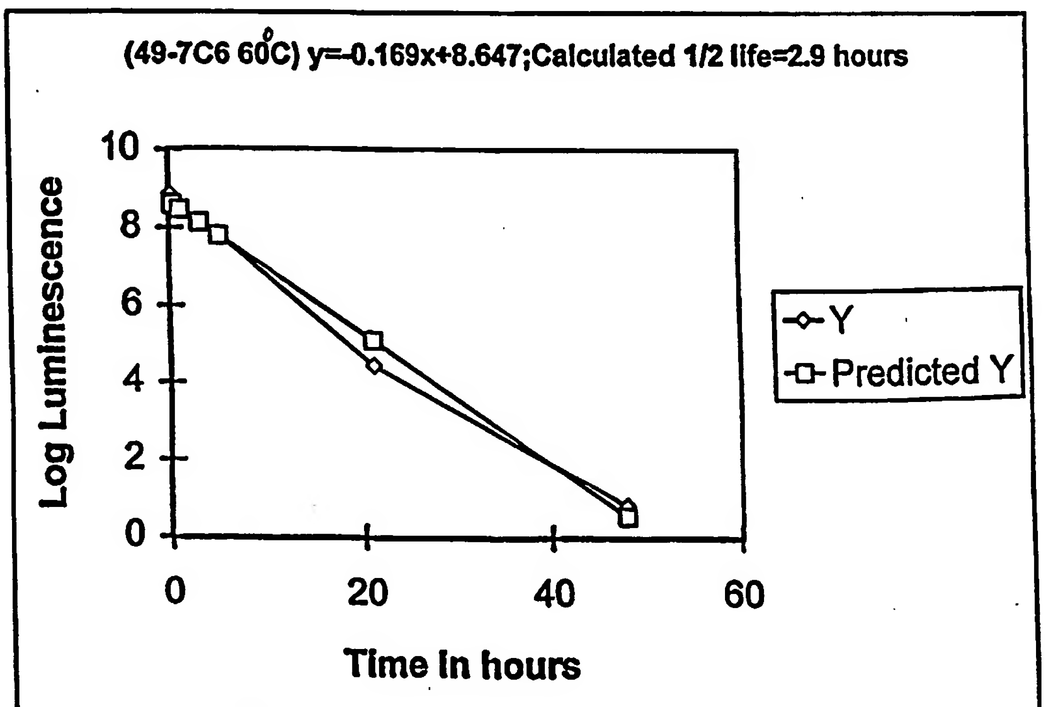

(49-7C6 60°C) y=-0.169x+8.647;Calculated 1/2 life=2.9 hours
Log Luminescence
10
8
6
4
2
0
0
20
40
60
Time in hours
Y
Predicted Y

FIG. 8

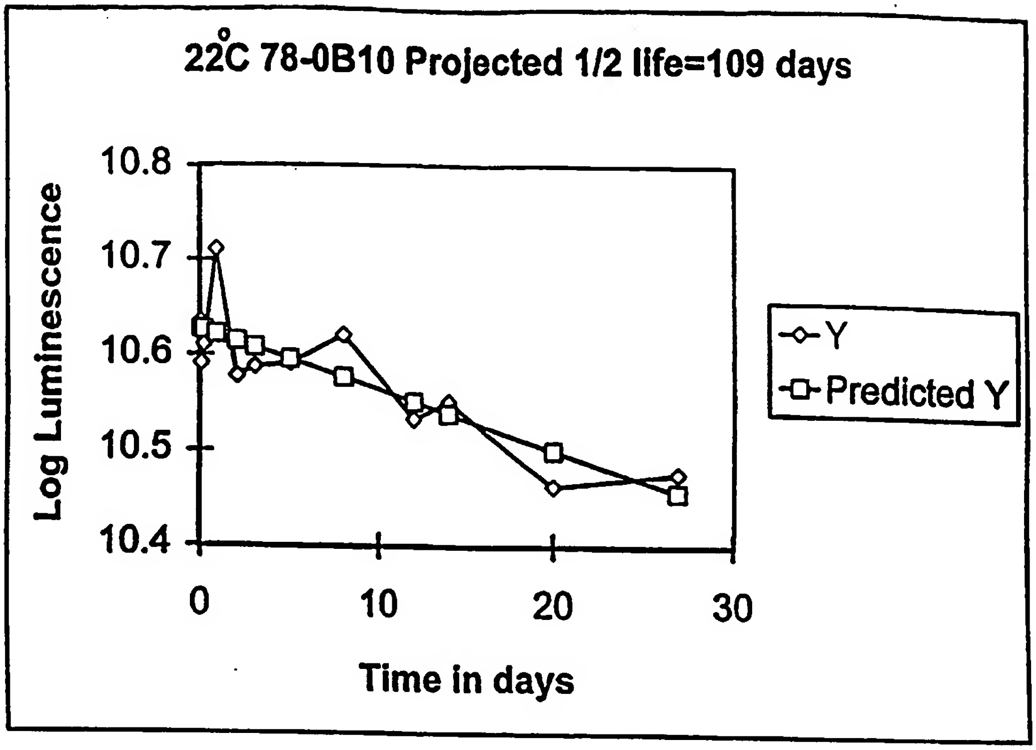

22°C 78-0B10 Projected 1/2 life=109 days
Log Luminescence
10.8
10.7
10.6
10.5
10.4
0
10
20
30
Time in days
Y
Predicted Y

FIG. 9

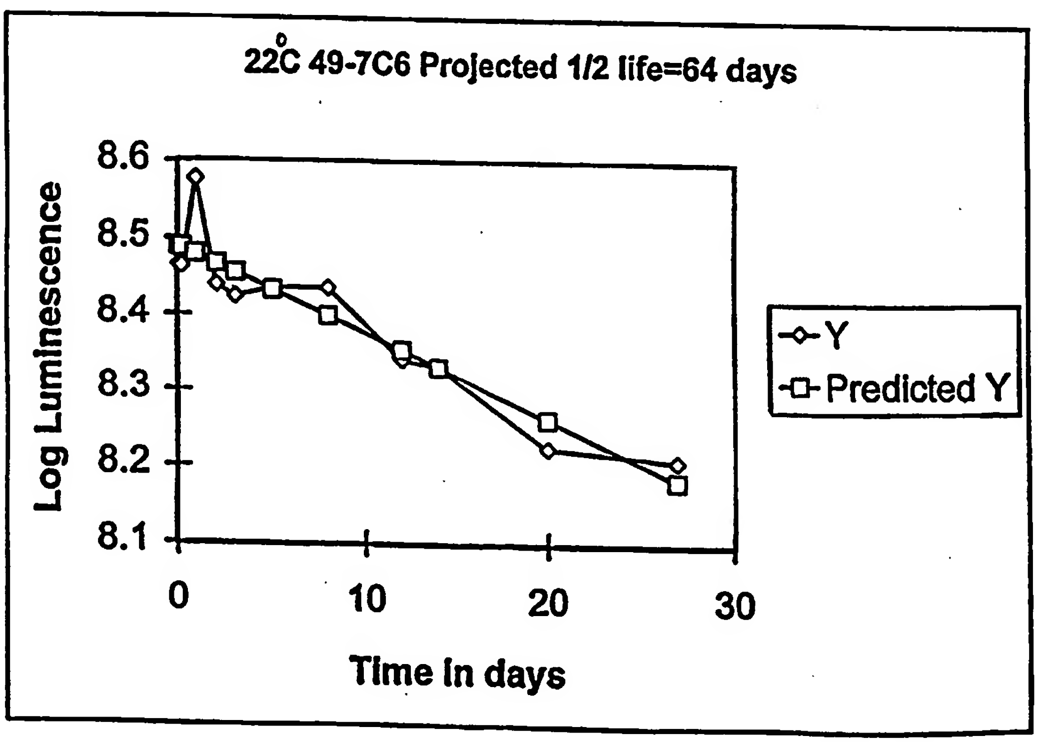

22°C 49-7C6 Projected 1/2 life=64 days
Log Luminescence
8.6
8.5
8.4
8.3
8.2
8.1
0
10
20
30
Time in days
Y
Predicted Y

FIG. 10

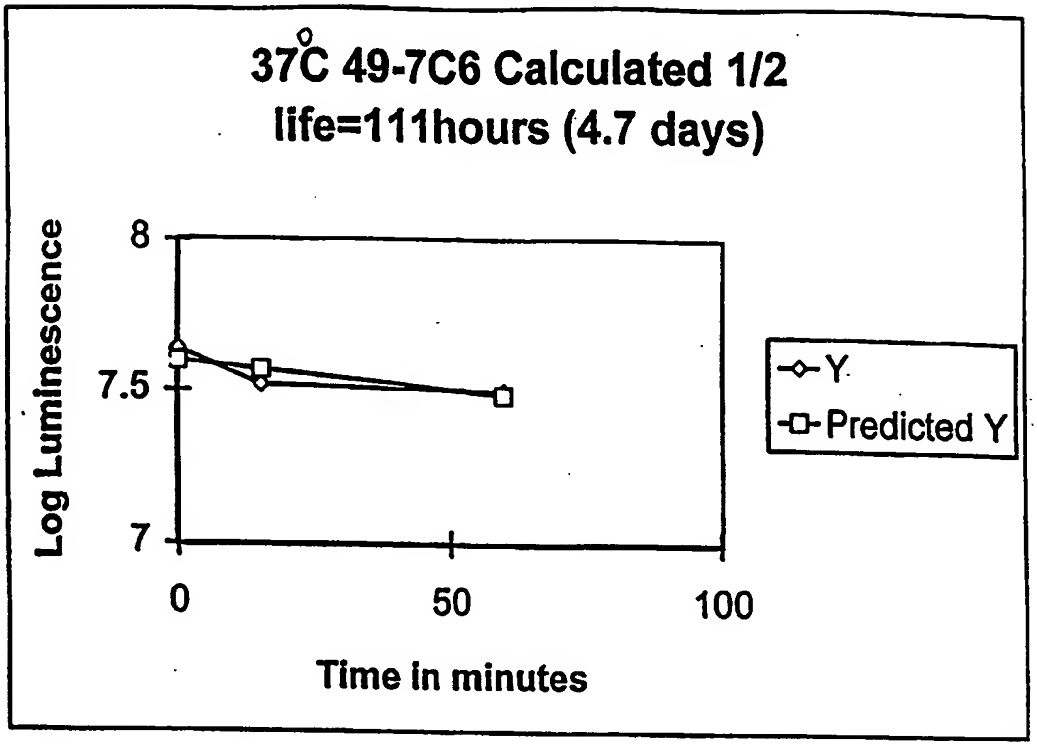

37°C 49-7C6 Calculated 1/2 life=111hours (4.7 days)
Log Luminescence
8
7.5
7
0
50
100
Time in minutes
Y
Predicted Y

FIG. 11

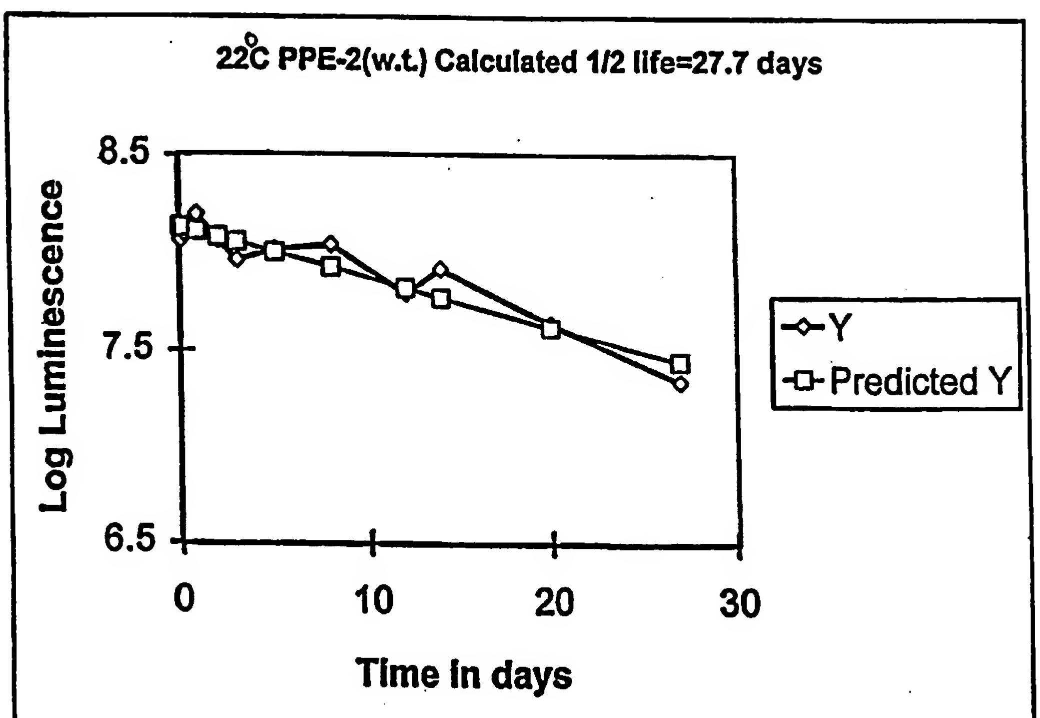

22°C PPE-2(w.t.) Calculated 1/2 life=27.7 days
Log Luminescence
8.5
7.5
6.5
0
10
20
30
Time in days
Y
Predicted Y

FIG. 12

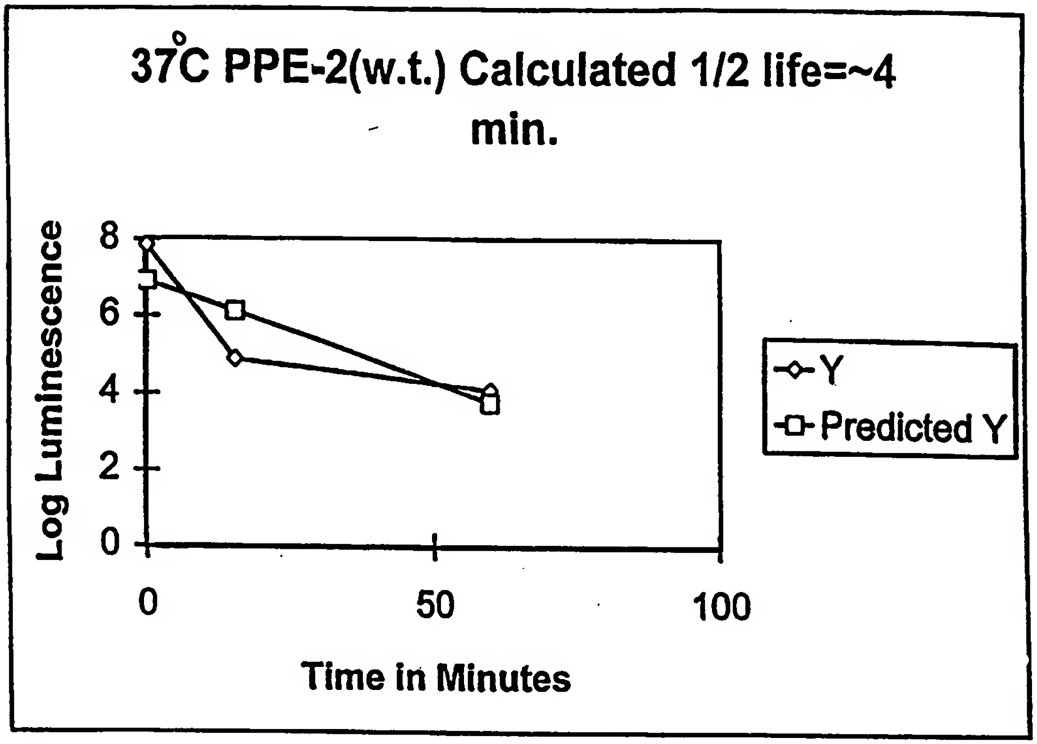

37°C PPE-2(w.t.) Calculated 1/2 life=~4 min.
Log Luminescence
8
6
4
2
0
0
50
100
Time in Minutes
Y
Predicted Y

FIG. 13

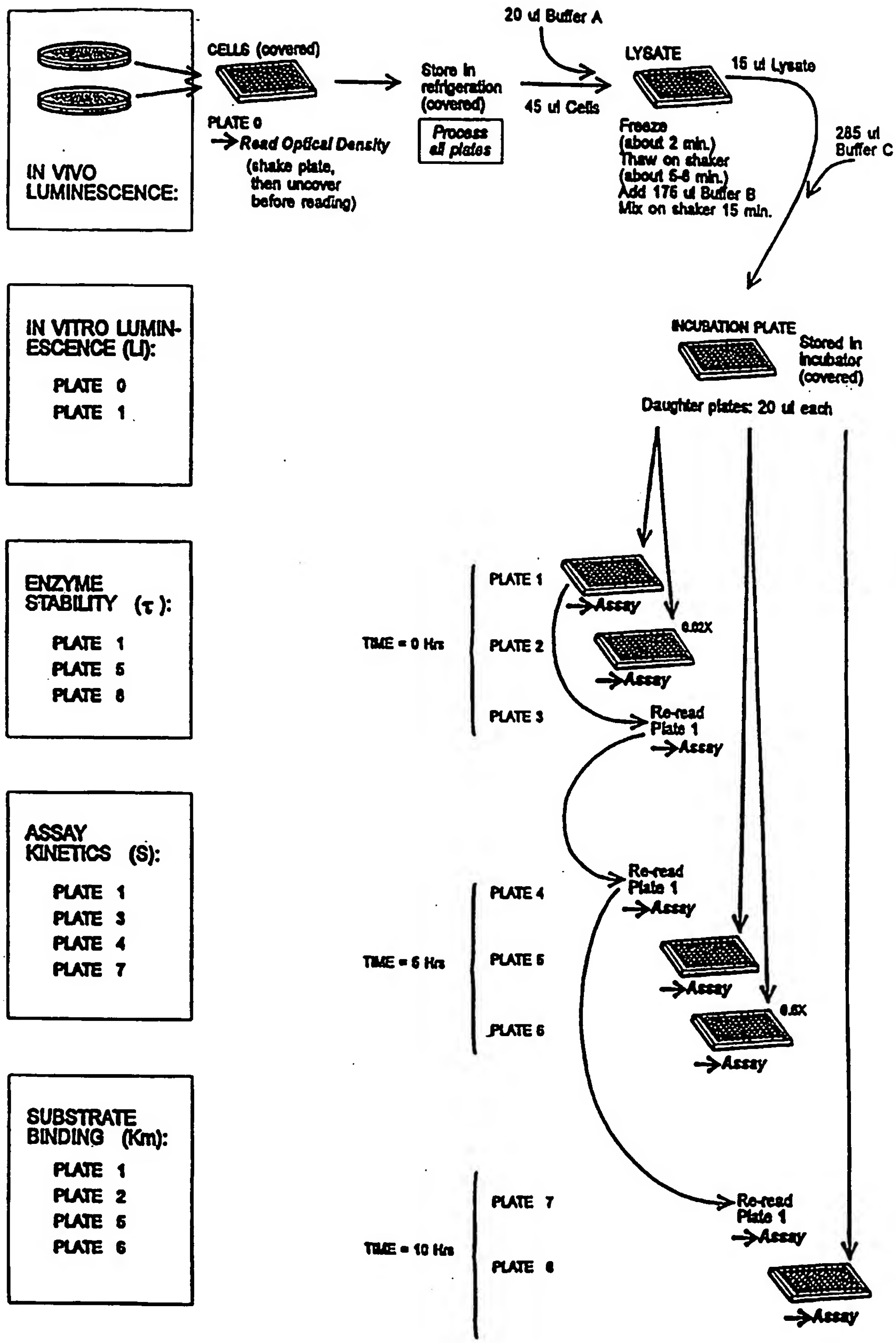
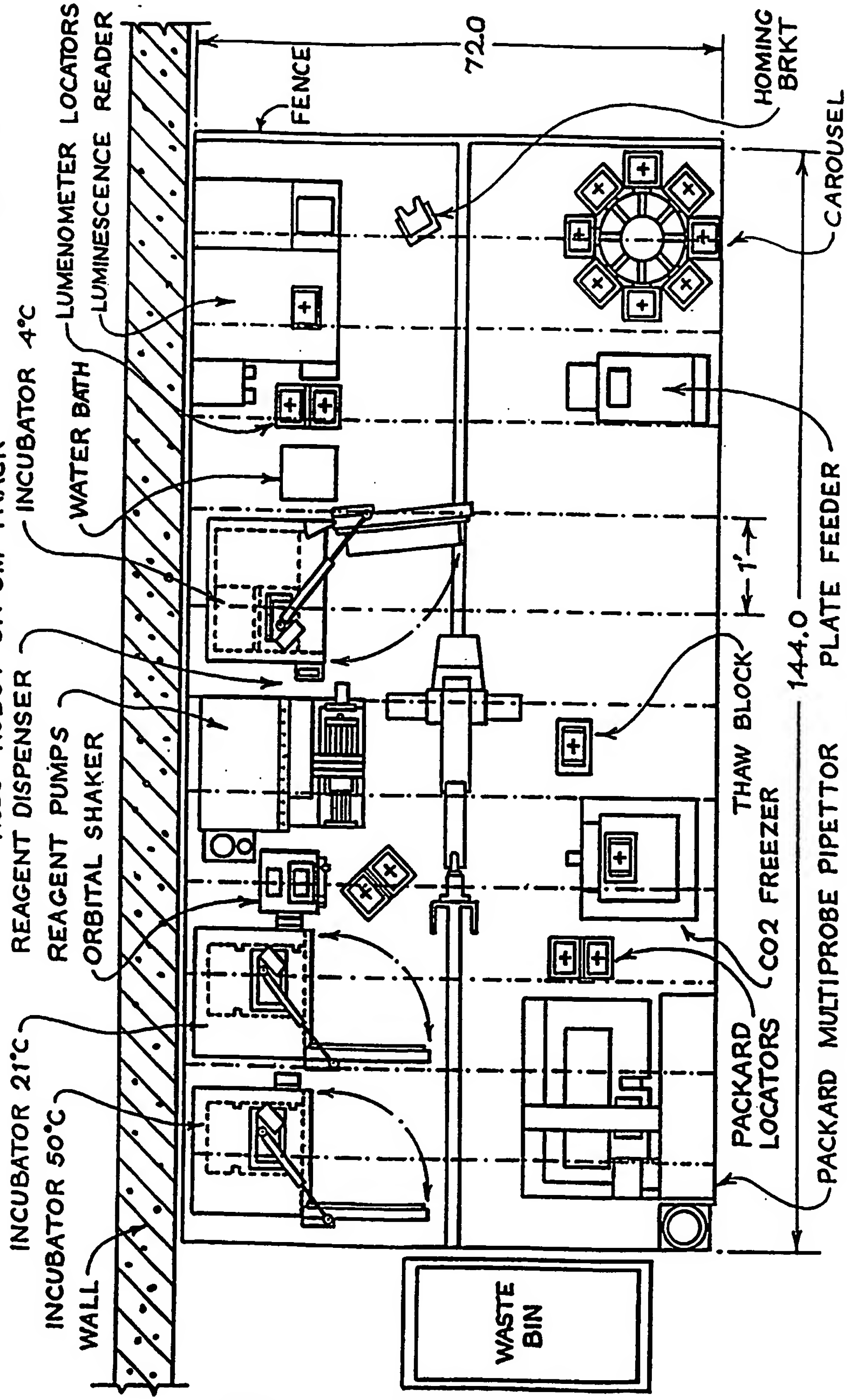

IN VIVO LUMINESCENCE:
CELLS (covered)
PLATE 0
→Read Optical Density (shake plate, then uncover before reading)
Store in refrigeration (covered)
Process all plates
20 ul Buffer A
45 ul Cells
LYSATE
15 ul Lysate
Freeze (about 2 min.) Thaw on shaker (about 5-8 min.) Add 175 ul Buffer B Mix on shaker 15 min.
285 ul Buffer C
IN VITRO LUMINESCENCE (LI):
PLATE 0
PLATE 1
INCUBATION PLATE
Stored in incubator (covered)
Daughter plates: 20 ul each
ENZYME STABILITY (τ):
PLATE 1
PLATE 5
PLATE 8
TIME = 0 Hrs
PLATE 1
PLATE 2
PLATE 3
→Assay
0.02X
→Assay
Re-read Plate 1
→Assay
ASSAY KINETICS (S):
PLATE 1
PLATE 3
PLATE 4
PLATE 7
Re-read Plate 1
→Assay
TIME = 5 Hrs
PLATE 4
PLATE 5
PLATE 6
→Assay
0.5X
→Assay
SUBSTRATE BINDING (Km):
PLATE 1
PLATE 2
PLATE 5
PLATE 6
TIME = 10 Hrs
PLATE 7
PLATE 8
Re-read Plate 1
→Assay
→Assay

FIG. 14

FIG. 15

PROMEGA - TABLE TOP LAYOUT
T265 ROBOT ON 3M TRACK



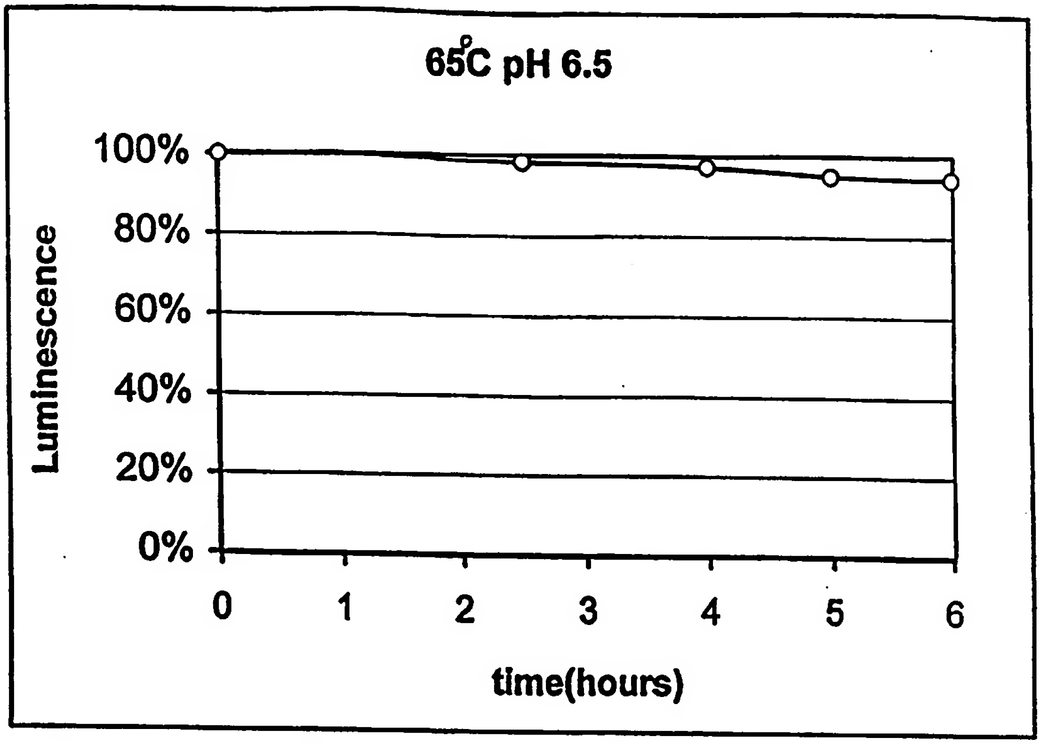


FIG. 16A

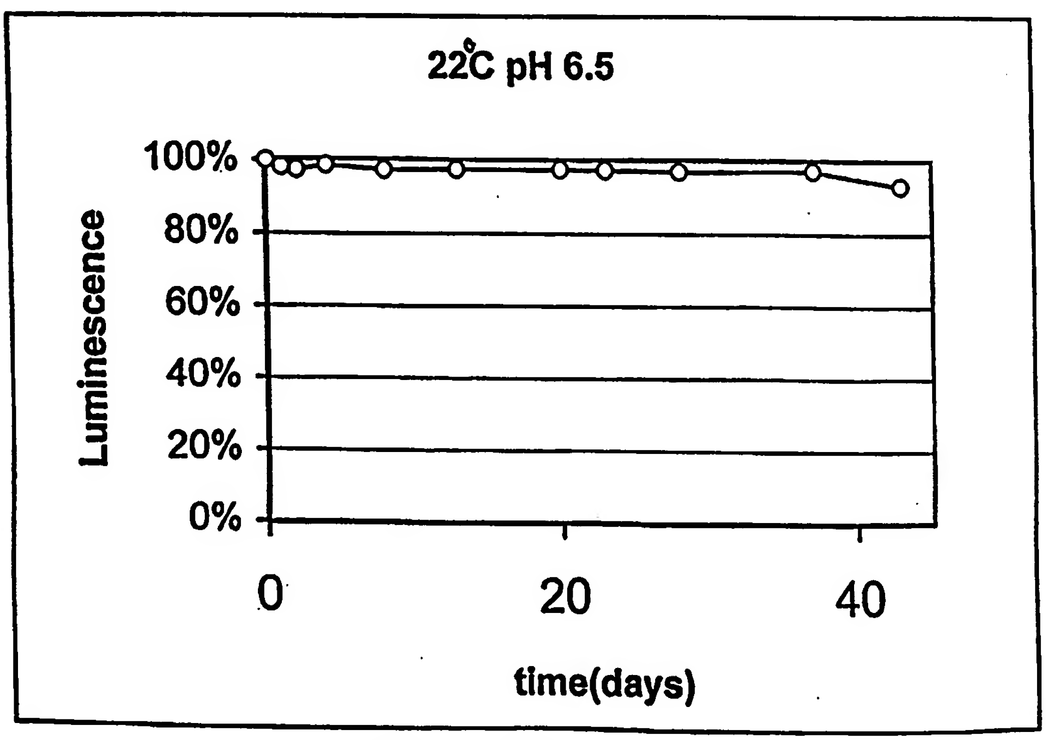


FIG. 16B

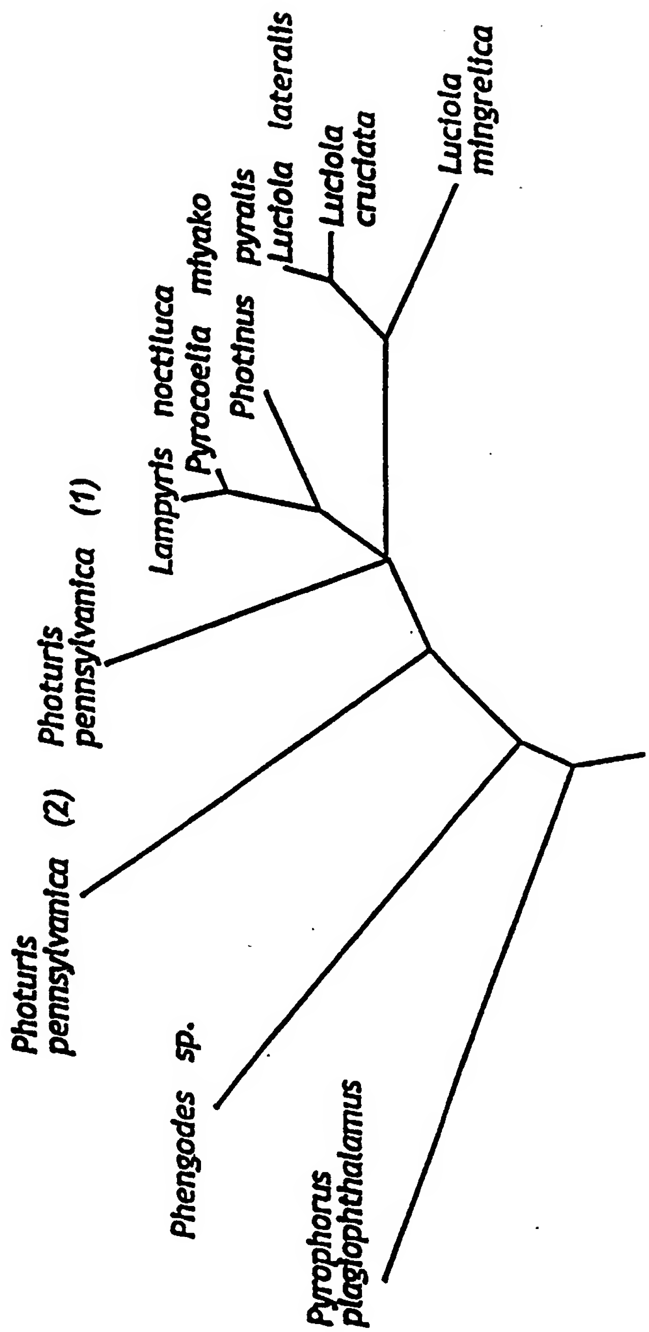

Luciola lateralis
Luciola mingrelica
Luciola cruciata
Pyrocoelia miyako
Photinus pyralis
Lampyris noctiluca
Photuris pennsylvanica (1)
Photuris pennsylvanica (2)
Phengodes sp.
Pyrophorus plagiophthalamus

FIG. 17

FIG. 18A

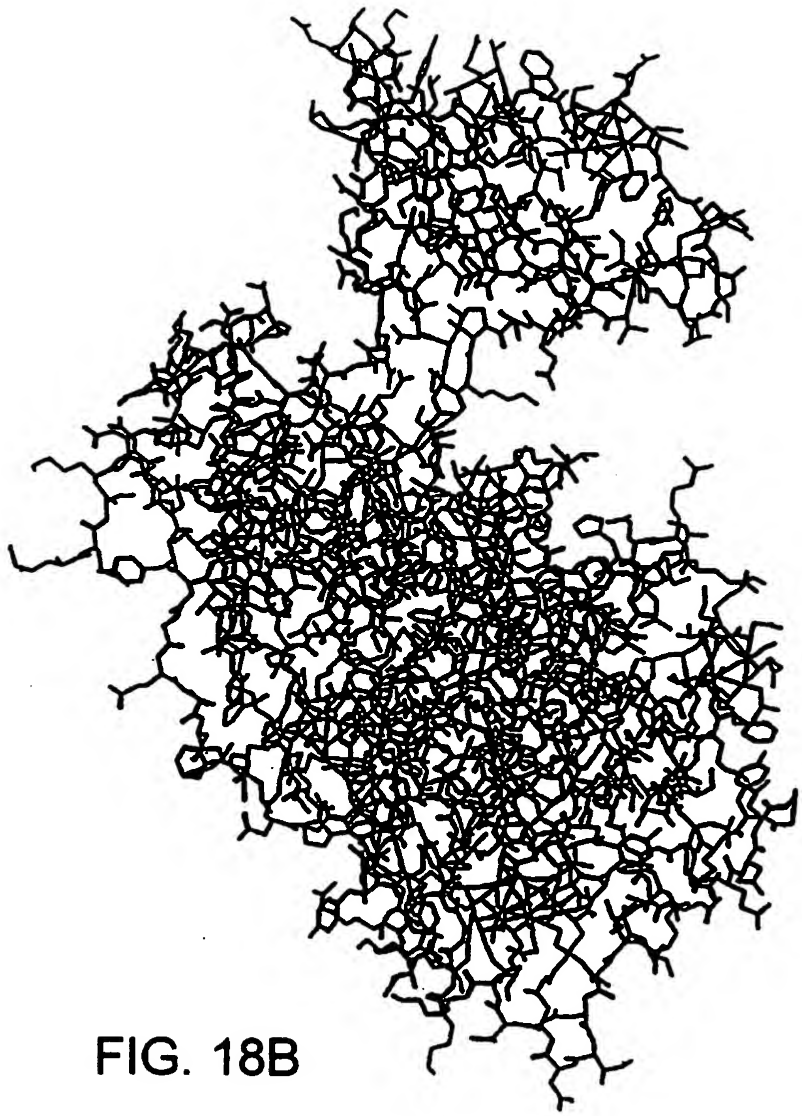


FIG. 18B

FIG. 18C

```
          1                                                 50
Lcr       MENMENDE.N IVVGPKPFYP IEEGSAGTQL RKYMERYAKL .GAIAFTNAV
Lla       MENMENDE.N IVYGPEPFYP IEEGSAGAQL RKYMDRYAKL .GAIAFTNAL
Lmi       ME.MEKEE.N VVYGPLPFYP IEEGSAGIQL HKYMHQYAKL .GAIAFSNAL
Pmi       ...MEDDSKH IMHGHRHSIL WEDGTAGEQL HKAMKRYAQV PGTIAFTDAH
Ppy       ...MED.AKN IKKGPAPFYP LEDGTAGEQL HKAMKRYALV PGTIAFTDAH
Lno       ...MED.AKN IMHGPAPFYP LEDGTAGEQL HKAMKRYAQV PGTIAFTDAH
Ppe1      ...MSI.ENN ILIGPPPYYP LEEGTAGEQL HRAISRYAAV PGTLAYTDVH
Phg       MIKME..EEH VMPGAMPRDL LFEGTAGQQL HRALYKHSYF PE..AIVDSH
GR        ...MMKREKN VVYGPEPLHP LEDLTAGEML FRALRKHSHL PQ..ALVDVY
YG        ...MMKREKN VIYGPEPLHP LEDLTAGEML FRALRKHSHL PQ..ALVDVF
Ppe2      ...MED..KN ILYGPEPFYP LADGTAGEQM FYALSRYADI SGCIALTNAH
49-7C6        A
78-0B10       A                             D
90-1B5        A                  E          D          P
133-1B2       A                  E          D          P
146-1H2       A                  E          D       A  P
Cons      ---M------ ---G------ -----AG--- ---------- ----A-----

          51                                                100
Lcr       TGVDYSYAEY LEKSCCLGKA LQNYGLVVDG RIALCSENCE EFFIPVIAGL
Lla       TGVDYTYAEY LEKSCCLGEA LKNYGLVVDG RIALCSENCE EFFIPVLAGL
Lmi       TGVDISYQEY FDITCRLAEA MKNFGMKPEE HIALCSENCE EFFIPVLAGL
Pmi       AEVNITYSEY FEMSCRLAET MKRYGLGLQH HIAVCSETSL QFFMPVCGAL
Ppy       IEVNITYAEY FEMSVRLAEA MKRYGLNTNH RIVVCSENSL QFFMPVLGAL
Lno       AEVNITYSEY FEMACRLAET MKRYGLGLQH HIAVCSENSL QFFMPVCGAL
Ppe1      TELEVTYKEF LDVTCRLAEA MKNYGLGLQH TISVCSENCV QFFMPICAAL
Phg       THEIISYAKI LDMSCRLAVS FQKYGLTQNN IIGICSENNL NFFNPVIAAF
GR        GEEWISYKEF FETTCLLAQS LHNCGYKMSD VVSICAENNK RFFVPIIAAW
YG        GDESLSYKEF FEATCLLAQS LHNCGYKMND VVSICAENNK RFFIPIIAAW
Ppe2      TKENVLYEEF LKLSCRLAES FKKYGLKQND TIAVCSENGL QFFLPLIASL
49-7C6                                                    I
78-0B10                                                   V
90-1B5                                                    V
133-1B2                                           S       V
146-1H2                                           S       V
Cons      ------Y--- ------L--- ----G----- ----C-E--- -FF-P-----

          101                                               150
Lcr       FIGVGVAPTN EIYTLRELVH SLGISKPTIV FSSKKGLDKV ITVQKTVTTI
Lla       FIGVGVAPTN EIYTLRELVH SLGISKPTIV FSSKKGLDKV ITVQKTVATI
Lmi       YIGVAVAPTN EIYTLRELNH SLGIAQPTIV FSSRKGLPKV LEVQKTVTCI
Pmi       FIGVGVAPTN DIYNERELYN SLFISQPTIV FCSKRALQKI LGVQKKLPVI
Ppy       FIGVAVAPAN DIYNERELLN SMNISQPTVV FVSKKGLQKI LNVQKKLPII
Lno       FIGVGVASTN DIYNERELYN SLSISQPTIV SCSKRALQKI LGVQKKLPII
Ppe1      YVGVATAPTN DIYNERELYN SLSISQPTVV FTSRNSLQKI LGVQSRLPII
Phg       YLGITVATVN DTYTDRELSE TLNITKPQML FCSKQSLPIV MKTMKIMPYV
GR        YIGMIVAPVN EGYIPDELCK VMGISRPQLV FCTKNILNKV LEVQSRTDFI
YG        YIGMIVAPVN ESYIPDELCK VMGISKPQIV FCTKNILNKV LEVQSRTNFI
Ppe2      YLGIIAAPVS DKYIERELIH SLGIVKPRII FCSKNTFQKV LNVKSKLKYV
49-7C6
78-0B10                                                     S
90-1B5         V   N                     V                  SI
133-1B2        V   N                     V                  SI
146-1H2        V   N                     V                  SI
Cons      --G---A--- --Y---EL-- ---I--P--- ---------- ----------
```

FIG. 19A

```
         151                                                200
Lcr      KTIVILDSKV DYRGYQCLDT FIKRNTPPGF QASSFKTVEV .DRKEQVALI
Lla      KTIVILDSKV DYRGYQSMDN FIKKNTPQGF KSSSFKTVEV .NRKEQVALI
Lmi      KKIVILDSKV NFGGHDCMET FIKKHVELGF QPSSFVPIDV KNRKQHVALL
Pmi      QKIVILDSRE DYMGKQSMYS FIESHLPAGF NEYDYIPDSF .DRETATALI
Ppy      QKIIIMDSKT DYQGFQSMYT FVTSHLPPGF NEYDFVPESF .DRDKTIALI
Lno      QKIVILDSRE DYMGKQSMYS FIESHLPAGF NEYDYIPDSF .DRETATALI
Ppe1     KKIIILDGKK DYLGYQSMQS FMKEHVPANF NVSAFKPLSF .DLDR.VACI
Phg      QKLLIIDSMQ DIGGIECVHS FVSRYTDEHF DPLKFVPLDF .DPREQVALI
GR       KRIIILDAVE NIHGCESLPN FISRYSDGNI A..NFKPLHY .DPVEQVAAI
YG       KRIIILDTVE NIHGCESLPN FISRYSDGNI A..NFKPLHY .DPVEQVAAI
Ppe2     ETIIILDLNE DLGGYQCLNN FISQNSDINL DVKKFKPNSF .NRDDQVALV
49-7C6                                          Y
78-0B10                             S           Y
90-1B5                              S           Y            I
133-1B2           D                 S           Y            I
146-1H2                             S           Y           SI
Cons     ----I-D--- ---G------ F--------- ---------- -------A--

         201                                                250
Lcr      MNSSGSTGLP KGVQLTHENT VTRFSHARDP IYGNQVSPGT AVLTVVPFHH
Lla      MNSSGSTGLP KGVQLTHENA VTRFSHARDP IYGNQVSPGT AILTVVPFHH
Lmi      MNSSGSTGLP KGVRITHEGA VTRFSHAKDP IYGNQVSPGT AILTVVPFHH
Pmi      MNSSGSTGLP KGVDLTHMNV CVRFSHCRDP VFGNQIIPDT AILTVIPFHH
Ppy      MNSSGSTGLP KGVALPHRTA CVRFSHARDP IFGNQIIPDT AILSVVPFHH
Lno      MNSSGSTGLP KGVELTHQNV CVRFSHCRDP VFGNQIIPDT AILTVIPFHH
Ppe1     MNSSGSTGLP KGVPISHRNT IYRFSHCRDP VFGNQIIPDT TILCAVPFHH
Phg      MTSSGTTGLP KGVMLTHRNI CVRFVHSRDP LFGTRFIPET SILSLVPFHH
GR       LCSSGTTGLP KGVMQTHRNV CVRLIHALDP RVGTQLIPGV TVLVYLPFFH
YG       LCSSGTTGLP KGVMQTHQNI CVRLIHALDP RAGTQLIPGV TVLVYLPFFH
Ppe2     MFSSGTTGVS KGVMLTHKNI VARFSHCKDP TFGNAINPTT AILTVIPFHH
49-7C6                                LA
78-0B10           P                   LA
90-1B5           LP                   LA
133-1B2          LP                   IA             S
146-1H2          LP                   IA             S
Cons     --SSG-TG-- KGV---H--- --R--H--DP --G----P-- --L---PF-H

         251                                                300
Lcr      GFGMFTTLGY LICGFRVVML TKFDEETFLK TLQDYKCTSV ILVPTLFAIL
Lla      GFGMFTTLGY LTCGFRIVML TKFDEETFLK TLQDYKCSSV ILVPTLFAIL
Lmi      GFGMFTTLGY FACGYRVVML TKFDEELFLR TLQDYKCTSV ILVPTLFAIL
Pmi      VFQMFTTLGY LTCGFRIVLM YRFEEELFLR SLQDYKIQSA LLVPTLFSFF
Ppy      GFGMFTTLGY LICGFRVVLM YRFEEELFLR SLQDYKIQSA LLVPTLFSFF
Lno      GFGMFTTLGY LTCGFRIVLM YRFEEELFLR SLQDYKIQSA LLVPTLFSFF
Ppe1     AFGTFTNLGY LICGFHVVLM YRFNEHLFLQ TLQDYKCQSA LLVPTVLAFL
Phg      AFGMFTTLSY FIVGLKIVMM KRFDGELFLK TIQNYKIPTI VIAPPVMVFL
GR       AFGFSINLGY FMVGLRVIML RRFDQEAFLK AIQDYEVRSV INVPAIILFL
YG       AFGFSINLGY FMVGLRVIML RRFDQEAFLK AIQDYEVRSV INVPAIILFL
Ppe2     GFGMTTTLGY FTCGFRVALM HTFEEKLFLQ SLQDYKVEST LLVPTLMAFF
49-7C6       M             V                                 L
78-0B10      M             V                                 L
90-1B5       M             V                                 L
133-1B2      M             V                                 L
146-1H2      M             V                                 L
Cons     -F-----L-Y ---G------ --F----FL- --Q-Y----- ---P------
```

FIG. 19A (Continued)

```
         301                                                350
Lcr      NKSELLNKYD LSNLVEIASG GAPLSKEVGE AVARRFNLPG VRQGYGLTET
Lla      NRSELLDKYD LSNLVEIASG GAPLSKEIGE AVARRFNLPG VRQGYGLTET
Lmi      NKSELIDKFD LSNLTEIASG GAPLAKEVGE AVARRFNLPG VRQGYGLTET
Pmi      AKSTLVDKYD LSNLHEIASG GAPLAKEVGE AVAKRFKLPG IRQGYGLTET
Ppy      AKSTLIDKYD LSNLHEIASG GAPLSKEVGE AVAKRFHLPG IRQGYGLTET
Lno      AKSTLVDKYD LSNLHEIASG GAPLAKEVGE AVAKRFKLPG IRQGYGLTET
Ppe1     AKNPLVDKYD LSNLHEIASG GAPLSKEISE IAAKRFKLPG IRQGYGLTET
Phg      AKSHLVDKYD LSSIKEIATG GAPLGPALAN AVAKRLKLGG IIQGYGLTET
GR       SKSPLVDKYD LSSLRELCCG AAPLAKEVAE IAVKRLNLPG IRCGFGLTES
YG       SKSPLVDKYD LSSLRELCCG AAPLAKEVAE VAVKRLNLPG IRCGFGLTES
Ppe2     AKSALVEKYD LSHLKEIASG GAPLSKEIGE MVKKRFKLNF VRQGYGLTET
49-7C6
78-0B10
90-1B5
133-1B2
146-1H2
Cons     ----L--K-D LS---E---G -APL------ ----R--L-- ---G-GLTE-

         351                                                400
Lcr      TSAIIITPEG DDKPGASGKV VPLFKAKVID LDTKKSLGPN RRGEVCVKGP
Lla      TSAIIITPEG DDKPGASGKV VPLFKAKVID LDTKKTLGPN RRGEVCVKGP
Lmi      TSAFIITPEG DDKPGASGKV VPLFKVKVID LDTKKTLGVN RRGEICVKGP
Pmi      TSAIIITPEG DDKPGACGKV VPFFTAKIVD LDTGKTLGVN QRGELCVKGP
Ppy      TSAILITPEG DDKPGAVGKV VPFFEAKVVD LDTGKTLGVN QRGELCVRGP
Lno      TSAIIITPEG DDKPGACGKV VPFFSAKIVD LDTGKTLGVN QRGELCVKGP
Ppe1     TCAIVITAEG EFKLGAVGKV VPFYSLKVLD LNTGKKLGPN ERGEICFKGP
Phg      CCAVLITPHN KIKTGSTGQV LPYVTAKIVD TKTGKNLGPN QTGELCFKSD
GR       TSANIHSLRD EFKSGSLGRV TPLMAAKIAD RETGKALGPN QVGELCIKGP
YG       TSANIHSLGD EFKSGSLGRV TPLMAAKIAD RETGKALGPN QVGELCVKGP
Ppe2     TSAVLITPDT DVRPGSTGKI VPFHAVKVVD PTTGKILGPN ETGELYFKGD
49-7C6           NN                                P
78-0B10          KG A                              P        A
90-1B5           KG AK                             P        P
133-1B2          KG AK                             P        P
146-1H2          KG AK         L                   P        P
Cons     --A------- ----G--G-- -P----K--D --T-K-LG-N --GE------

         401                                                450
Lcr      MLMKGYVNNP EATKELIDEE GWLHTGDIGY YDEEKHFFIV DRLKSLIKYK
Lla      MLMKGYVDNP EATREIIDEE GWLHTGDIGY YDEEKHFFIV DRLKSLIKYK
Lmi      SLMLGYSNNP EATRETIDEE GWLHTGDIGY YDEDEHFFIV DRLKSLIKYK
Pmi      MIMKGYVNNP EATNALIDKD GWLHSGDIAY YDKDGHFFIV DRLKSLIKYK
Ppy      MIMSGYVNNP EATNALIDKD GWLHSGDIAY WDEDEHFFIV DRLKSLIKYK
Lno      MIMKGYVNNP EATSALIDKD GWLHSGDIAY YDKDGHFFIV DRLKSLIKYK
Ppe1     MIMKGYINNP EATRELIDEE GWIHSGDIGY FDEDGHVYIV DRLKSLIKYK
Phg      IIMKGYYQNE EETRLVIDKD GWLHSGDIGY YDTDGNFHIV DRLKELIKYK
GR       MVSKGYVNNV EATKEAIDDD GWLHSGDFGY YDEDEHFYVV DRYKELIKYK
YG       MVSKGYVNNV EATKEAIDDD GWLHSGDFGY YDEDEHFYVV DRYKELIKYK
Ppe2     MIMKSYYNNE EATKAIINKD GWLRSGDIAY YDNDGHFYIV DRLKSLIKYK
49-7C6       G
78-0B10      G                DN
90-1B5       G                DN
133-1B2      G                DN
146-1H2      G                DN
Cons     -----Y--N- E-T---I--- GW---GD--Y -D-------V DR-K-LIKYK
```

FIG. 19A (Continued)

```
         451                                                500
Lcr      GYQVPPAELE SVLLQHPSIF DAGVAGVPDP VAGELPGAVV VLESGKNMTE
Lla      GYQVPPAELE SVLLQHPNIF DAGVAGVPDP IAGELPGAVV VLEKGKSMTE
Lmi      GYQVPPAELE SVLLQHPNIF DAGVAGVPDP DAGELPGAVV VMEKGKTMTE
Pmi      GYQVPPAELE SILLQHPFIF DAGVAGIPDP DAGELPAAVV VLEEGKMMTE
Ppy      GYQVAPAELE SILLQHPNIF DAGVAGLPDD DAGELPAAVV VLEHGKTMTE
Lno      GYQVPPAELE SILLQHPFIF DAGVAGIPDP DAGELPAAVV VLEEGKTMTE
Ppe1     GYQVPPAELE ALLLQHPFIE DAGVAGVPDE VAGDLPGAVV VLKEGKSITE
Phg      AYQVAPAELE ALLLQHPYIA DAGVTGIPDE EAGELPAACV VLEPGKTMTE
GR       GSQVAPAELE EILLKNPCIR DVAVVGIPDL EAGELPSAFV VIQPGKEITA
YG       GSQVAPAELE EILLKNPCIR DVAVVGIPDL EAGELPSAFV VKQPGKEITA
Ppe2     GYQVAPAEIE GILLQHPYIV DAGVTGIPDE AAGELPAAGV VVQTGKYLNE
49-7C6
78-0B10
90-1B5
133-1B2
146-1H2
Cons     --QV-PAE-E --LL--P-I- D--V-G-PD- -AG-LP-A-V V---GK----

         501                                                550
Lcr      KEVMDYVASQ VSNAKRLRGG VRFVDEVPKG LTGKIDGRA. IREILKKPV.
Lla      KEVMDYVASQ VSNAKRLRGG VRFVDEVPKG LTGKIDGKA. IREILKKPV.
Lmi      KEIVDYVNSQ VVNHKRLRGG VRFVDEVPKG LTGKIDAKV. IREILKKPQ.
Pmi      QEVMDYVAGQ VTASKRLRGG VKFVDEVPKG LTGKIDSRK. IREILTMGQK
Ppy      KEIVDYVASQ VTTAKKLRGG VVFVDEVPKG LTGKLDARK. IREILIKAKK
Lno      QEVMDYVAGQ VTASKRLRGG VKFVDEVPKG LTGKIDGRK. IREILMMGKK
Ppe1     KEIQDYVAGQ VTSSKKLRGG VEFVKEVPKG FTGKIDTRK. IKEILIKAQK
Phg      KEVMDYIAER VTPTKRLRGG VLFVNNIPKG ATGKLVRTE. LRRLLTQRA.
GR       KEVYDYLAER VSHTKYLRGG VRFVDSIPRN VTGKITRKEL LKQLLEKS..
YG       KEVYDYLAER VSHTKYLRGG VRFVDSIPRN VTGKITRKEL LKQLLEKS..
Ppe2     QIVQNFVSSQ VSTAKWLRGG VKFLDEIPKG STGKIDRKV. LRQMFEKH..
49-7C6
78-0B10      D
90-1B5       DY A
133-1B2      DY A                 I                         L
146-1H2      DY A                                           L
Cons     ---------- V---K-LRGG V-F----P-- -TGK------ ----------

         551
Lcr      ......AKM
Lla      ......AKM
Lmi      ......AKM
Pmi      ......SKL
Ppy      G...GKSKL
Lno      ......SKL
Ppe1     GKSKSKAKL
Phg      ......AKL
GR       ......SKL
YG       ......SKL
Ppe2     .....KSKL
49-7C6         TNG*
78-0B10        TNG*
90-1B5         TNG*
133-1B2        TNG*
146-1H2        TNG*
```

FIG. 19A (Continued)

Lcr:	Luciola cruciata	Phg:	Phengodes sp.
Lla:	Luciola lateralis	Lmi:	Luciola mingrelica
Gr:	Pyrophorus plagiophthalamus (green)		
YG:	Pyrophorus plagiophthalamus (yellow green)		
Pmi:	Pyrocoelia miyako	Ppy:	Photinus pyralis
Lno:	Lampyris noctiluca		
Ppe-2:	Photuris pennsylvanica (2)		
Ppe-1:	Photuris pennsylvanica (1)		
Cons:	Sites which are strictly conserved		

FIG. 19A (Continued)

	1				50
Lcr	MENMENDE.N	IVVGPKPFYP	IEEGSAGTQL	RKYMERYAKL	.GAIAFTNAV
Lla	MENMENDE.N	IVYGPEPFYP	IEEGSAGAQL	RKYMDRYAKL	.GAIAFTNAL
Lmi	ME.MEKEE.N	VVYGPLPFYP	IEEGSAGIQL	HKYMHQYAKL	.GAIAFSNAL
Pmi	...MEDDSKH	IMHGHRHSIL	WEDGTAGEQL	HKAMKRYAQV	PGTIAFTDAH
Ppy	...MED.AKN	IKKGPAPFYP	LEDGTAGEQL	HKAMKRYALV	PGTIAFTDAH
Lno	...MED.AKN	IMHGPAPFYP	LEDGTAGEQL	HKAMKRYAQV	PGTIAFTDAH
Ppe1	...MSI.ENN	ILIGPPPYYP	LEEGTAGEQL	HRAISRYAAV	PGTLAYTDVH
Ppe2	...MED..KN	ILYGPEPFYP	LADGTAGEQM	FYALSRYADI	SGCIALTNAH
Phg	MIKME..EEH	VMPGAMPRDL	LFEGTAGQQL	HRALYKHSYF	PE..AIVDSH
GR	...MMKREKN	VVYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVY
YG	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVF
30-4B02	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVV
81-6G01	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVV
Cons	---M------	---G------	-----AG---	----------	----A-----

	51				100
Lcr	TGVDYSYAEY	LEKSCCLGKA	LQNYGLVVDG	RIALCSENCE	EFFIPVIAGL
Lla	TGVDYTYAEY	LEKSCCLGEA	LKNYGLVVDG	RIALCSENCE	EFFIPVLAGL
Lmi	TGVDISYQEY	FDITCRLAEA	MKNFGMKPEE	HIALCSENCE	EFFIPVLAGL
Pmi	AEVNITYSEY	FEMSCRLAET	MKRYGLGLQH	HIAVCSETSL	QFFMPVCGAL
Ppy	IEVNITYAEY	FEMSVRLAEA	MKRYGLNTNH	RIVVCSENSL	QFFMPVLGAL
Lno	AEVNITYSEY	FEMACRLAET	MKRYGLGLQH	HIAVCSENSL	QFFMPVCGAL
Ppe1	TELEVTYKEF	LDVTCRLAEA	MKNYGLGLQH	TISVCSENCV	QFFMPICAAL
Ppe2	TKENVLYEEF	LKLSCRLAES	FKKYGLKQND	TIAVCSENGL	QFFLPLIASL
Phg	THEIISYAKI	LDMSCRLAVS	FQKYGLTQNN	IIGICSENNL	NFFNPVIAAF
GR	GEEWISYKEF	FETTCLLAQS	LHNCGYKMSD	VVSICAENNK	RFFVPIIAAW
YG	GDESLSYKEF	FEATCLLAQS	LHNCGYKMND	VVSICAENNK	RFFIPIIAAW
30-4B02	GDESLSYKEF	FEATVLLAQS	LHNCGYKMND	VVSICAENNK	RFFIPVIAAW
81-6G01	GDESLSYKEF	FEATVLLAQS	LHNCGYKMND	VVSICAENNT	RFFIPVIAAW
Cons	------Y---	------L---	----G-----	----C-E---	-FF-P-----

	101				150
Lcr	FIGVGVAPTN	EIYTLRELVH	SLGISKPTIV	FSSKKGLDKV	ITVQKTVTTI
Lla	FIGVGVAPTN	EIYTLRELVH	SLGISKPTIV	FSSKKGLDKV	ITVQKTVATI
Lmi	YIGVAVAPTN	EIYTLRELNH	SLGIAQPTIV	FSSRKGLPKV	LEVQKTVTCI
Pmi	FIGVGVAPTN	DIYNERELYN	SLFISQPTIV	FCSKRALQKI	LGVQKKLPVI
Ppy	FIGVAVAPAN	DIYNERELLN	SMNISQPTVV	FVSKKGLQKI	LNVQKKLPII
Lno	FIGVGVASTN	DIYNERELYN	SLSISQPTIV	SCSKRALQKI	LGVQKKLPII
Ppe1	YVGVATAPTN	DIYNERELYN	SLSISQPTVV	FTSRNSLQKI	LGVQSRLPII
Ppe2	YLGIIAAPVS	DKYIERELIH	SLGIVKPRII	FCSKNTFQKV	LNVKSKLKYV
Phg	YLGITVATVN	DTYTDRELSE	TLNITKPQML	FCSKQSLPIV	MKTMKIMPYV
GR	YIGMIVAPVN	EGYIPDELCK	VMGISRPQLV	FCTKNILNKV	LEVQSRTDFI
YG	YIGMIVAPVN	ESYIPDELCK	VMGISKPQIV	FCTKNILNKV	LEVQSRTNFI
30-4B02	YIGMIVAPVN	ESYIPDELCK	VMGISKPQIV	FTTKNILNKV	LEVQSRTNFI
81-6G01	YIGMIVAPVN	ESYIPDELCK	VMGISKPQIV	FTTKNILNKV	LEVQSRTNFI
Cons	--G---A---	--Y---EL--	---I--P---	----------	----------

FIG. 19B

	151				200
Lcr	KTIVILDSKV	DYRGYQCLDT	FIKRNTPPGF	QASSFKTVEV	.DRKEQVALI
Lla	KTIVILDSKV	DYRGYQSMDN	FIKKNTPQGF	KGSSFKTVEV	.NRKEQVALI
Lmi	KKIVILDSKV	NFGGHDCMET	FIKKHVELGF	QPSSFVPIDV	KNRKQHVALL
Pmi	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppy	QKIIIMDSKT	DYQGFQSMYT	FVTSHLPPGF	NEYDFVPESF	.DRDKTIALI
Lno	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppe1	KKIIILDGKK	DYLGYQSMQS	FMKEHVPANF	NVSAFKPLSF	.DLDR.VACI
Ppe2	ETIIILDLNE	DLGGYQCLNN	FISQNSDINL	DVKKFKPNSF	.NRDDQVALV
Phg	QKLLIIDSMQ	DIGGIECVHS	FVSRYTDEHF	DPLKFVPLDF	.DPREQVALI
GR	KRIIILDAVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVAAI
YG	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVAAI
30-4B02	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVAAI
81-6G01	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHF	.DPVEQVAAI
Cons	*----I-D---*	*---G------*	*F---------*	*----------*	*-------A--*

	201				250
Lcr	MNSSGSTGLP	KGVQLTHENT	VTRFSHARDP	IYGNQVSPGT	AVLTVVPFHH
Lla	MNSSGSTGLP	KGVQLTHENA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH
Lmi	MNSSGSTGLP	KGVRITHEGA	VTRFSHAKDP	IYGNQVSPGT	AILTVVPFHH
Pmi	MNSSGSTGLP	KGVDLTHMNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppy	MNSSGSTGLP	KGVALPHRTA	CVRFSHARDP	IFGNQIIPDT	AILSVVPFHH
Lno	MNSSGSTGLP	KGVELTHQNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppe1	MNSSGSTGLP	KGVPISHRNT	IYRFSHCRDP	VFGNQIIPDT	TILCAVPFHH
Ppe2	MFSSGTTGVS	KGVMLTHKNI	VARFSHCKDP	TFGNAINPTT	AILTVIPFHH
Phg	MTSSGTTGLP	KGVMLTHRNI	CVRFVHSRDP	LFGTRFIPET	SILSLVPFHH
GR	LCSSGTTGLP	KGVMQTHRNV	CVRLIHALDP	RVGTQLIPGV	TVLVYLPFFH
YG	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
30-4B02	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
81-6G01	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
Cons	*--SSG-TG--*	*KGV---H---*	*--R--H--DP*	*--G----P--*	*--L---PF-H*

	251				300
Lcr	GFGMFTTLGY	LICGFRVVML	TKFDEETFLK	TLQDYKCTSV	ILVPTLFAIL
Lla	GFGMFTTLGY	LTCGFRIVML	TKFDEETFLK	TLQDYKCSSV	ILVPTLFAIL
Lmi	GFGMFTTLGY	FACGYRVVML	TKFDEELFLR	TLQDYKCTSV	ILVPTLFAIL
Pmi	VFQMFTTLGY	LTCGFRIVLM	YRFEEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppy	GFGMFTTLGY	LICGFRVVLM	YRFEEELFLR	SLQDYKIQSA	LLVPTLFSFF
Lno	GFGMFTTLGY	LTCGFRIVLM	YRFEEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppe1	AFGTFTNLGY	LICGFHVVLM	YRFNEHLFLQ	TLQDYKCQSA	LLVPTVLAFL
Ppe2	GFGMTTTLGY	FTCGFRVALM	HTFEEKLFLQ	SLQDYKVEST	LLVPTLMAFF
Phg	AFGMFTTLSY	FIVGLKIVMM	KRFDGELFLK	TIQNYKIPTI	VIAPPVMVFL
GR	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
YG	AFGPSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
30-4B02	AFGPSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
81-6G01	AFGPSITLGY	FMVGLRVIMF	RRFDQEAFLK	AIQDYEVRSV	INVPSVILFL
Cons	*-F-----L-Y*	*---G------*	*--F----FL-*	*--Q-Y-----*	*---P------*

FIG. 19B (Continued)

```
              301                                                  350
     Lcr   NKSELLNKYD LSNLVEIASG GAPLSKEVGE AVARRFNLPG VRQGYGLTET
     Lla   NRSELLDKYD LSNLVEIASG GAPLSKEIGE AVARRFNLPG VRQGYGLTET
     Lmi   NKSELIDKFD LSNLTEIASG GAPLAKEVGE AVARRFNLPG VRQGYGLTET
     Pmi   AKSTLVDKYD LSNLHEIASG GAPLAKEVGE AVAKRFKLPG IRQGYGLTET
     Ppy   AKSTLIDKYD LSNLHEIASG GAPLSKEVGE AVAKRFHLPG IRQGYGLTET
     Lno   AKSTLVDKYD LSNLHEIASG GAPLAKEVGE AVAKRFKLPG IRQGYGLTET
    Ppe1   AKNPLVDKYD LSNLHEIASG GAPLSKEISE IAAKRFKLPG IRQGYGLTET
    Ppe2   AKSALVEKYD LSHLKEIASG GAPLSKEIGE MVKKRFKLNF VRQGYGLTET
     Phg   AKSHLVDKYD LSSIKEIATG GAPLGPALAN AVAKRLKLGG IIQGYGLTET
      GR   SKSPLVDKYD LSSLRELCCG AAPLAKEVAE IAVKRLNLPG IRCGFGLTES
      YG   SKSPLVDKYD LSSLRELCCG AAPLAKEVAE VAVKRLNLPG IRCGFGLTES
 30-4B02   SKSPLVDKYD LSSLRELCCG AAPLAKEVAE VAAKRLNLPG IRCGFGLTES
 81-6G01   SKSPLVDKYD LSSLRELCCG AAPLAKEVAE VAAKRLNLPG IRCGFGLTES
    Cons   ----L--K-D LS---E---G -APL------ ----R--L-- ---G-GLTE-

              351                                                  400
     Lcr   TSAIIITPEG DDKPGASGKV VPLFKAKVID LDTKKSLGPN RRGEVCVKGP
     Lla   TSAIIITPEG DDKPGASGKV VPLFKAKVID LDTKKTLGPN RRGEVCVKGP
     Lmi   TSAFIITPEG DDKPGASGKV VPLFKVKVID LDTKKTLGVN RRGEICVKGP
     Pmi   TSAIIITPEG DDKPGACGKV VPFFTAKIVD LDTGKTLGVN QRGELCVKGP
     Ppy   TSAILITPEG DDKPGAVGKV VPFFEAKVVD LDTGKTLGVN QRGELCVRGP
     Lno   TSAIIITPEG DDKPGACGKV VPFFSAKIVD LDTGKTLGVN QRGELCVKGP
    Ppe1   TCAIVITAEG EFKLGAVGKV VPFYSLKVLD LNTGKKLGPN ERGEICFKGP
    Ppe2   TSAVLITPDT DVRPGSTGKI VPFHAVKVVD PTTGKILGPN ETGELYFKGD
     Phg   CCAVLITPEN KIKTGSTGQV LPYVTAKIVD TKTGKNLGPN QTGELCFKSD
      GR   TSANIHSLRD EFKSGSLGRV TPLMAAKIAD RETGKALGPN QVGELCIKGP
      YG   TSANIHSLGD EFKSGSLGRV TPLMAAKIAD RETGKALGPN QVGELCVKGP
 30-4B02   TSANIHSLRD EFKSGSIGRV TPLMAAKIAD RETGKALGPN QVGELCIKGP
 81-6G01   TSANIHSLRD EFKSGSLGRV TPLMAAKIAD RETGKALGPN QVGELCIKGP
    Cons   --A------- ----G--G-- -P----K--D --T-K-LG-N --GE------

              401                                                  450
     Lcr   MLMKGYVNNP EATKELIDEE GWLHTGDIGY YDEEKHFFIV DRLKSLIKYK
     Lla   MLMKGYVDNP EATREIIDEE GWLHTGDIGY YDEEKHFFIV DRLKSLIKYK
     Lmi   SLMLGYSNNP EATRETIDEE GWLHTGDIGY YDEDEHFFIV DRLKSLIKYK
     Pmi   MIMKGYVNNP EATNALIDKD GWLHSGDIAY YDKDGHFFIV DRLKSLIKYK
     Ppy   MIMSGYVNNP EATNALIDKD GWLHSGDIAY WDEDEHFFIV DRLKSLIKYK
     Lno   MIMKGYVNNP EATSALIDKD GWLHSGDIAY YDKDGHFFIV DRLKSLIKYK
    Ppe1   MIMKGYINNP EATRELIDEE GWIHSGDIGY FDEDGHVYIV DRLKSLIKYK
    Ppe2   MIMKSYYNNE EATKAIINKD GWLRSGDIAY YDNDGHFYIV DRLKSLIKYK
     Phg   IIMKGYYQNE EETRLVIDKD GWLHSGDIGY YDTDGNFHIV DRLKELIKYK
      GR   MVSKGYVNNV EATKEAIDDD GWLHSGDFGY YDEDEHFYVV DRYKELIKYK
      YG   MVSKGYVNNV EATKEAIDDD GWLHSGDFGY YDEDEHFYVV DRYKELIKYK
 30-4B02   MVSKGYVNNV EATKEAIDDD GWLHSGDFGY YDEDEHFYVV DRYKELIKYK
 81-6G01   MVSKGYVNNV EATKEAIDDD GWLHSGDFGY YDEDEHFYVV DRYKELIKYK
    Cons   -----Y--N- E-T---I--- GW---GD--Y -D-------V DR-K-LIKYK
```

FIG. 19B (Continued)

	451				500
Lcr	GYQVPPAELE	SVLLQHPSIF	DAGVAGVPDP	VAGELPGAVV	VLESGKNMTE
Lla	GYQVPPAELE	SVLLQHPNIF	DAGVAGVPDP	IAGELPGAVV	VLEKGKSMTE
Lmi	GYQVPPAELE	SVLLQHPNIF	DAGVAGVPDP	DAGELPGAVV	VMEKGKTMTE
Pmi	GYQVPPAELE	SILLQHPFIF	DAGVAGIPDP	DAGELPAAVV	VLEEGKMMTE
Ppy	GYQVAPAELE	SILLQHPNIF	DAGVAGLPDD	DAGELPAAVV	VLEHGKTMTE
Lno	GYQVPPAELE	SILLQHPFIF	DAGVAGIPDP	DAGELPAAVV	VLEEGKTMTE
Ppe1	GYQVPPAELE	ALLLQHPFIE	DAGVAGVPDE	VAGDLPGAVV	VLKEGKSITE
Ppe2	GYQVAPAEIE	GILLQHPYIV	DAGVTGIPDE	AAGELPAAGV	VVQTGKYLNE
Phg	AYQVAPAELE	ALLLQHPYIA	DAGVTGIPDE	EAGELPAACV	VLEPGKTMTE
GR	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VIQPGKEITA
YG	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
30-4B02	GSQVAPAELE	EILLTNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
81-6G01	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
Cons	*--QV-PAE-E*	*--LL--P-I-*	*D--V-G-PD-*	*-AG-LP-A-V*	*V---GK----*

	501				550
Lcr	KEVMDYVASQ	VSNAKRLRGG	VRFVDEVPKG	LTGKIDGRA.	IREILKKPV.
Lla	KEVMDYVASQ	VSNAKRLRGG	VRFVDEVPKG	LTGKIDGKA.	IREILKKPV.
Lmi	KEIVDYVNSQ	VVNHKRLRGG	VRFVDEVPKG	LTGKIDAKV.	IREILKKPQ.
Pmi	QEVMDYVAGQ	VTASKRLRGG	VKFVDEVPKG	LTGKIDSRK.	IREILTMGQK
Ppy	KEIVDYVASQ	VTTAKKLRGG	VVFVDEVPKG	LTGKLDARK.	IREILIKAKK
Lno	QEVMDYVAGQ	VTASKRLRGG	VKFVDEVPKG	LTGKIDGRK.	IREILMMGKK
Ppe1	KEIQDYVAGQ	VTSSKKLRGG	VEFVKEVPKG	FTGKIDTRK.	IKEILIKAQK
Ppe2	QIVQNFVSSQ	VSTAKWLRGG	VKFLDEIPKG	STGKIDRKV.	LRQMFEKH..
Phg	KEVMDYIAER	VTPTKRLRGG	VLFVNNIPKG	ATGKLVRTE.	LRRLLTQRA.
GR	KEVYDYLAER	VSHTKYLRGG	VRFVDSIPRN	VTGKITRKEL	LKQLLEKS..
YG	KEVYDYLAER	VSHTKYLRGG	VRFVDSIPRN	VTGKITRKEL	LKQLLEKS..
30-4B02	KEVYDYLAER	VSHTKYLRGG	VRFVDSIPRN	VTGKITRKEL	LKQLLEK...
81-6G01	KEVYDYLAER	VSHTKYLRGG	VRFVDSIPRN	VTGKITRKEL	LKQLLEK...
Cons	*----------*	*V---K-LRGG*	*V-F----P--*	*-TGK------*	*----------*

	551
Lcr	......AKM
Lla	......AKM
Lmi	......AKM
Pmi	......SKL
Ppy	G...GKSKL
Lno	......SKL
Ppe1	GKSKSKAKL
Ppe2	.....KSKL
Phg	......AKL
GR	......SKL
YG	......SKL
30-4B02	.....AGG*
81-6G01	.....AGG*
Cons	*-------K-*

FIG. 19B (Continued)

```
             1                                                      50
     Lcr    MENMENDE.N IVVGPKPFYP IEEGSAGTQL RKYMERYAKL .GAIAFTNAV
     Lla    MENMENDE.N IVYGPEPFYP IEEGSAGAQL RKYMDRYAKL .GAIAFTNAL
     Lmi    ME.MEKEE.N VVYGPLPFYP IEEGSAGIQL HKYMHQYAKL .GAIAFSNAL
     Pmi    ...MEDDSKH INHGHRHSIL WEDGTAGEQL HKAMKRYAQV PGTIAFTDAH
     Ppy    ...MED.AKN IKKGPAPFYP LEDGTAGEQL HKAMKRYALV PGTIAFTDAH
     Lno    ...MED.AKN IMHGPAPFYP LEDGTAGEQL HKAMKRYAQV PGTIAFTDAH
     Ppe1   ...MSI.ENN ILIGPPPYYP LEEGTAGEQL HRAISRYAAV PGTLAYTDVH
     Ppe2   ...MED..KN ILYGPEPFYP LADGTAGEQM FYALSRYADI SGCIALTNAH
     Phg    MIKME..EEH VMPGAMPRDL LFEGTAGQQL HRALYKHSYF PE..AIVDSH
     YG     ...MMKREKN VIYGPEPLHP LEDLTAGEML FRALRKHSHL PQ..ALVDVF
            ---XXOOOOO XOO-OOOXXO OOXXO--OXO XOOXOXXXOO OXOO-XXOXX
```

```
             51                                                    100
     Lcr    TGVDYSYAEY LEKSCCLGKA LQNYGLVVDG RIALCSENCE EFFIPVIAGL
     Lla    TGVDYTYAEY LEKSCCLGEA LKNYGLVVDG RIALCSENCE EFFIPVLAGL
     Lmi    TGVDISYQEY FDITCRLAEA MKNFGMKPEE HIALCSENCE EFFIPVLAGL
     Pmi    AEVNITYSEY FEMSCRLAET MKRYGLGLQH HIAVCSETSL QFFMPVCGAL
     Ppy    IEVNITYAEY FEMSVRLAEA MKRYGLNTNH RIVVCSENSL QFFMPVLGAL
     Lno    AEVNITYSEY FEMACRLAET MKRYGLGLQH HIAVCSENSL QFFMPVCGAL
     Ppe1   TELEVTYKEF LDVTCRLAEA MKNYGLGLQH TISVCSENCV QFFMPICAAL
     Ppe2   TKENVLYEEF LKLSCRLAES FKKYGLKQND TIAVCSENGL QFFLPLIASL
     Phg    THEIISYAKI LDNSCRLAVS FQKYGLTQNN IIGICSENNL NFFNPVIAAF
     YG     GDESLSYKEF FEATCLLAQS LHNCGYKMND VVSICAENNK RFFIPIIAAW
            XOXOXX---X XOOXOXOOX- XXOX-XOOOO OXXO-X-OOX X--O-XOOOX
```

```
             101                                                   150
     Lcr    FIGVGVAPTN EIYTLRELVH SLGISKPTIV FSSKKGLDKV ITVQKTVTTI
     Lla    FIGVGVAPTN EIYTLRELVH SLGISKPTIV FSSKKGLDKV ITVQKTVATI
     Lmi    YIGVAVAPTN EIYTLRELNH SLGIAQPTIV FSSRKGLPKV LEVQKTVTCI
     Pmi    FIGVGVAPTN DIYNERELYN SLFISQPTIV FCSKRALQKI LGVQKKLPVI
     Ppy    FIGVAVAPAN DIYNERELLN SMNISQPTVV FVSKKGLQKI LNVQKKLPII
     Lno    FIGVGVASTN DIYNERELYN SLSISQPTIV SCSKRALQKI LGVQKKLPII
     Ppe1   YVGVATAPTN DIYNERELYN SLSISQPTVV FTSRNSLQKI LGVQSRLPII
     Ppe2   YLGIIAAPVS DKYIERELIH SLGIVKPRII FCSKNTFQKV LNVKSKLKYV
     Phg    YLGITVATVN DTYTDRELSE TLNITKPQML FCSKQSLPIV MKTMKIMPYV
     YG     YIGMIVAPVN ESYIPDELCK VMGISKPQIV FCTKNILNKV LEVQSRTNFI
            XO-XOO-OXO XX-OXX--OO XXO-OX-XOO OOXOOOOXOO OOO-XOXXOO
```

FIG. 19C

	151				200
Lcr	KTIVILDSKV	DYRGYQCLDT	FIKRNTPPGF	QASSFKTVEV	.DRKEQVALI
Lla	KTIVILDSKV	DYRGYQSMDN	FIKKNTPQGF	KGSSFKTVEV	.NRKEQVALI
Lmi	KKIVILDSKV	NFGGHDCMET	FIKKHVELGF	QPSSFVPIDV	KNRKQHVALL
Pmi	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppy	QKIIIMDSKT	DYQGFQSMYT	FVTSHLPPGF	NEYDFVPESF	.DRDKTIALI
Lno	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppe1	KKIIILDGKK	DYLGYQSMQS	FMKEHVPANF	NVSAFKPLSF	.DLDR.VACI
Ppe2	ETIIILDLNE	DLGGYQCLNN	FISQNSDINL	DVKKFKPNSF	.NRDDQVALV
Phg	QKLLIIDSMQ	DIGGIECVHS	FVSRYTDEHF	DPLKFVPLDF	.DPREQVALI
YG	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVAAI
	OXOX-O-XXO	*XXO-OXOXOO*	*-OOOXOXOXX*	*O--OOOOOXX*	*-OXOOOO-XO*

	201				250
Lcr	MNSSGSTGLP	KGVQLTHENT	VTRFSHARDP	IYGNQVSPGT	AVLTVVPFHH
Lla	MNSSGSTGLP	KGVQLTHENA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH
Lmi	MNSSGSTGLP	KGVRITHEGA	VTRFSHAKDP	IYGNQVSPGT	AILTVVPFHH
Pmi	MNSSGSTGLP	KGVDLTHMNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppy	MNSSGSTGLP	KGVALPHRTA	CVRFSHARDP	IFGNQIIPDT	AILSVVPFHH
Lno	MNSSGSTGLP	KGVELTHQNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppe1	MNSSGSTGLP	KGVPISHRNT	IYRFSHCRDP	VFGNQIIPDT	TILCAVPFHH
Ppe2	MFSSGTTGVS	KGVMLTHKNI	VARFSHCKDP	TFGNAINPTT	AILTVIPFHH
Phg	MTSSGTTGLP	KGVMLTHRNI	CVRFVHSRDP	LFGTRFIPET	SILSLVPFHH
YG	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
	XX---X--OO	*---OXO-OOO*	*OO-XX-OX--*	*OX-XOXO-OX*	*XX-XXX--X-*

	251				300
Lcr	GFGMFTTLGY	LICGFRVVML	TKFDEETFLK	TLQDYKCTSV	ILVPTLFAIL
Lla	GFGMFTTLGY	LTCGFRIVML	TKFDEETFLK	TLQDYKCSSV	ILVPTLFAIL
Lmi	GFGMFTTLGY	FACGYRVVML	TKFDEELFLR	TLQDYKCTSV	ILVPTLFAIL
Pmi	VFQMFTTLGY	LTCGFRIVLM	YRFEEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppy	GFGMFTTLGY	LICGFRVVLM	YRFEEELFLR	SLQDYKIQSA	LLVPTLFSFF
Lno	GFGMFTTLGY	LTCGFRIVLM	YRFEEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppe1	AFGTFTNLGY	LICGFHVVLM	YRFNEHLFLQ	TLQDYKCQSA	LLVPTVLAFL
Ppe2	GFGMTTTLGY	FTCGFRVALM	HTFEEKLFLQ	SLQDYKVEST	LLVPTLMAFF
Phg	AFGMFTTLSY	FIVGLKIVMM	KRFDGELFLK	TIQNYKIPTI	VIAPPVMVFL
YG	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAILLFL
	X-OXXXX-O-	*XOX-XOOXXX*	*OO-OXOX--O*	*XX-O-XOOOO*	*XXO-XXXXOO*

FIG. 19C (Continued)

	301				350
Lcr	NKSELLNKYD	LSNLVEIASG	GAPLSKEVGE	AVARRFNLPG	VRQGYGLTET
Lla	NRSELLDKYD	LSNLVEIASG	GAPLSKEIGE	AVARRFNLPG	VRQGYGLTET
Lmi	NKSELIDKFD	LSNLTEIASG	GAPLAKEVGE	AVARRFNLPG	VRQGYGLTET
Pmi	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE	AVAKRFKLPG	IRQGYGLTET
Ppy	AKSTLIDKYD	LSNLHEIASG	GAPLSKEVGE	AVAKRFHLPG	IRQGYGLTET
Lno	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE	AVAKRFKLPG	IRQGYGLTET
Ppe1	AKNPLVDKYD	LSNLHEIASG	GAPLSKEISE	IAAKRFKLPG	IRQGYGLTET
Ppe2	AKSALVEKYD	LSHLKEIASG	GAPLSKEIGE	MVKKRFKLNF	VRQGYGLTET
Phg	AKSHLVDKYD	LSSIKEIATG	GAPLGPALAN	AVAKRLKLGG	IIQGYGLTET
YG	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE	VAVKRLNLPG	IRCGFGLTES
	XOOO-OO-O-	--XOO-XXX-	X---XOOOXO	XXXO-XX-OO	OOX-X----X

	351				400
Lcr	TSAIIITPEG	DDKPGASGKV	VPLFKAKVID	LDTKKSLGPN	RRGEVCVKGP
Lla	TSAIIITPEG	DDKPGASGKV	VPLFKAKVID	LDTKKTLGPN	RRGEVCVKGP
Lmi	TSAFIITPEG	DDKPGASGKV	VPLFKVKVID	LDTKKTLGVN	RRGEICVKGP
Pmi	TSAIIITPEG	DDKPGACGKV	VPFFTAKIVD	LDTGKTLGVN	QRGELCVKGP
Ppy	TSAILITPEG	DDKPGAVGKV	VPFFEAKVVD	LDTGKTLGVN	QRGELCVRGP
Lno	TSAIIITPEG	DDKPGACGKV	VPFFSAKIVD	LDTGKTLGVN	QRGELCVKGP
Ppe1	TCAIVITAEG	EFKLGAVGKV	VPFYSLKVLD	LNTGKKLGPN	ERGEICFKGP
Ppe2	TSAVLITPDT	DVRPGSTGKI	VPFHAVKVVD	PTTGKILGPN	ETGELYFKGD
Phg	CCAVLITPHN	KIKTGSTGQV	LPYVTAKIVD	TKTGKNLGPN	QTGELCFKSD
YG	TSANIHSLGD	EFKSGSLGRV	TPLMAAKIAD	RETGKALGPN	QVGELCVKGP
	OO-XOXXXXX	XXOX-XO-XO	X-XXOO-XX-	XX-O-X--O-	OX--OOOOOO

	401				450
Lcr	MLMKGYVNNP	EATKELIDEE	GWLHTGDIGY	YDEEKHFFIV	DRLKSLIKYK
Lla	MLMKGYVDNP	EATREIIDEE	GWLHTGDIGY	YDEEKHFFIV	DRLKSLIKYK
Lmi	SLMLGYSNNP	EATRETIDEE	GWLHTGDIGY	YDEDEHFFIV	DRLKSLIKYK
Pmi	MIMKGYVNNP	EATNALIDKD	GWLHSGDIAY	YDKDGHFFIV	DRLKSLIKYK
Ppy	MIMSGYVNNP	EATNALIDKD	GWLHSGDIAY	WDEDEHFFIV	DRLKSLIKYK
Lno	MIMKGYVNNP	EATSALIDKD	GWLHSGDIAY	YDKDGHFFIV	DRLKSLIKYK
Ppe1	MIMKGYINNP	EATRELIDEE	GWIHSGDIGY	FDEDGHVYIV	DRLKSLIKYK
Ppe2	MIMKSYYNNE	EATKAIINKD	GWLRSGDIAY	YDNDGHFYIV	DRLKSLIKYK
Phg	IIMKGYYQNE	EETRLVIDKD	GWLHSGDIGY	YDTDGNFHIV	DRLKELIKYK
YG	MVSKGYVNNV	EATKEAIDDD	GWLHSGDFGY	YDEDEHFYVV	DRYKELIKYK
	OXXOO-OO-X	-O-OOX-OXO	--OOO--XO-	O-OOXOOXX-	--X-X-----

FIG. 19C (Continued)

	451				500
Lcr	GYQVPPAELE	SVLLQHPSIF	DAGVAGVPDP	VAGELPGAVV	VLESGKNMTE
Lla	GYQVPPAELE	SVLLQHPNIF	DAGVAGVPDP	IAGELPGAVV	VLEKGKSMTE
Lmi	GYQVPPAELE	SVLLQHPNIF	DAGVAGVPDP	DAGELPGAVV	VMEKGKTMTE
Pmi	GYQVPPAELE	SILLQHPFIF	DAGVAGIPDP	DAGELPAAVV	VLEEGKMMTE
Ppy	GYQVAPAELE	SILLQHPNIF	DAGVAGLPDD	DAGELPAAVV	VLEHGKTMTE
Lno	GYQVPPAELE	SILLQHPFIF	DAGVAGIPDP	DAGELPAAVV	VLEEGKTMTE
Ppe1	GYQVPPAELE	ALLLQHPFIE	DAGVAGVPDE	VAGDLPGAVV	VLKEGKSITE
Ppe2	GYQVAPAEIE	GILLQHPYIV	DAGVTGIPDE	AAGELPAAGV	VVQTGKYLNE
Phg	AYQVAPAELE	ALLLQHPYIA	DAGVTGIPDE	EAGELPAACV	VLEPGKTMTE
YG	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
	OX--X---O-	XO--XX-O-X	-XX-X-O--X	O--O--X-X-	-XXO--OXOX

	501				550
Lcr	KEVMDYVASQ	VSNAKRLRGG	VRFVDEVPKG	LTGKIDGRA.	IREILKKPV.
Lla	KEVMDYVASQ	VSNAKRLRGG	VRFVDEVPKG	LTGKIDGKA.	IREILKKPV.
Lmi	KEIVDYVNSQ	VVNHKRLRGG	VRFVDEVPKG	LTGKIDAKV.	IREILKKPQ.
Pmi	QEVMDYVAGQ	VTASKRLRGG	VKFVDEVPKG	LTGKIDSRK.	IREILTMGQK
Ppy	KEIVDYVASQ	VTTAKKLRGG	VVFVDEVPKG	LTGKLDARK.	IREILIKAKK
Lno	QEVMDYVAGQ	VTASKRLRGG	VKFVDEVPKG	LTGKIDGRK.	IREILMMGKK
Ppe1	KEIQDYVAGQ	VTSSKKLRGG	VEFVKEVPKG	FTGKIDTRK.	IKEILIKAQK
Ppe2	QIVQNFVSSQ	VSTAKWLRGG	VKFLDEIPKG	STGKIDRKV.	LRQMFEKH..
Phg	KEVMDYIAER	VTPTKRLRGG	VLFVNNIPKG	ATGKLVRTE.	LRRLLTQRA.
YG	KEVYDYLAER	VSHTKYLRGG	VRFVDSIPRN	VTGKITRKEL	LKQLLEKS..
	OOOXOOXOXX	-OOO-X----	-O-OOXX-XX	X---OXOXOO	XXXXOOOOOO

	551
Lcr	......AKM
Lla	......AKM
Lmi	......AKM
Pmi	......SKL
Ppy	G...GKSKL
Lno	......SKL
Ppe1	GKSKSKAKL
Ppe2	.....KSKL
Phg	......AKL
YG	......SKL
	OOOOOOX-O

FIG. 19C (Continued)

Key to beetle luciferase sequences:

Lcr	*Luciola cruciata*
Lla	*Luciola lateralis*
Lmi	*Luciola mingrelica*
Pmi	*Pyrocoelia miyako*
Ppy	*Photinus pyralis*
Lno	*Lampyris noctiluca*
Ppe1	*Photuris pennsylvanica* (1)
Ppe2	*Photuris pennsylvanica* (2)
Phg	*Phengodes* sp.
YG	*Pyrophorus plagiophthalamus* – yellow-green luminescence

FIG. 19D

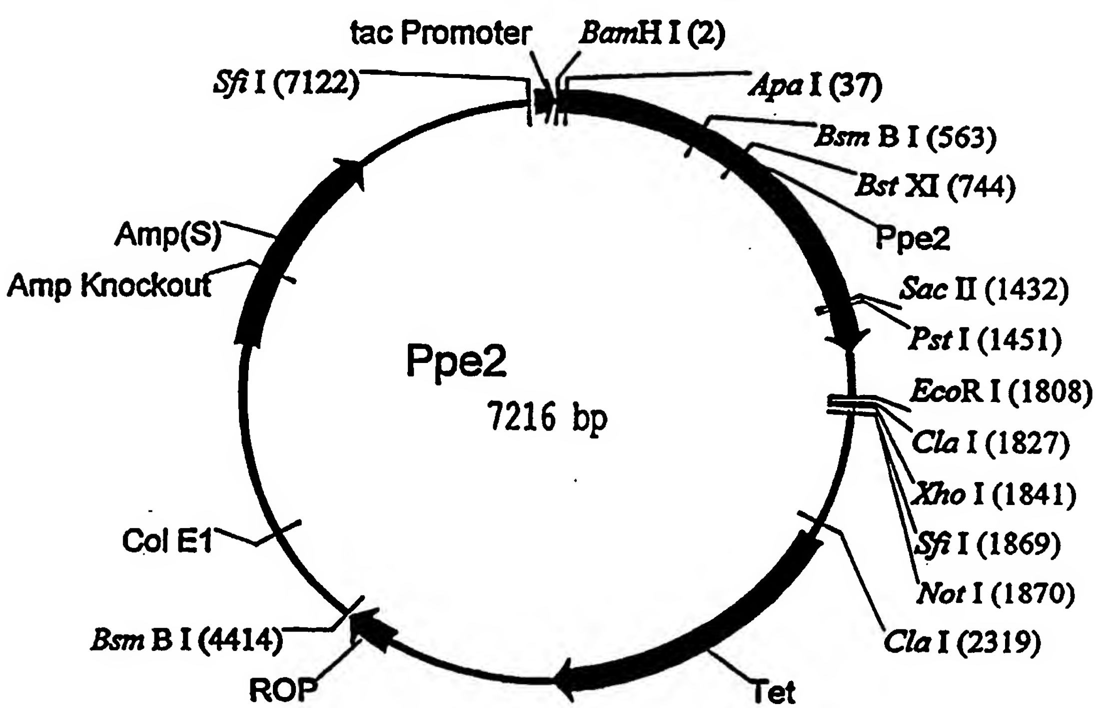


FIG. 20

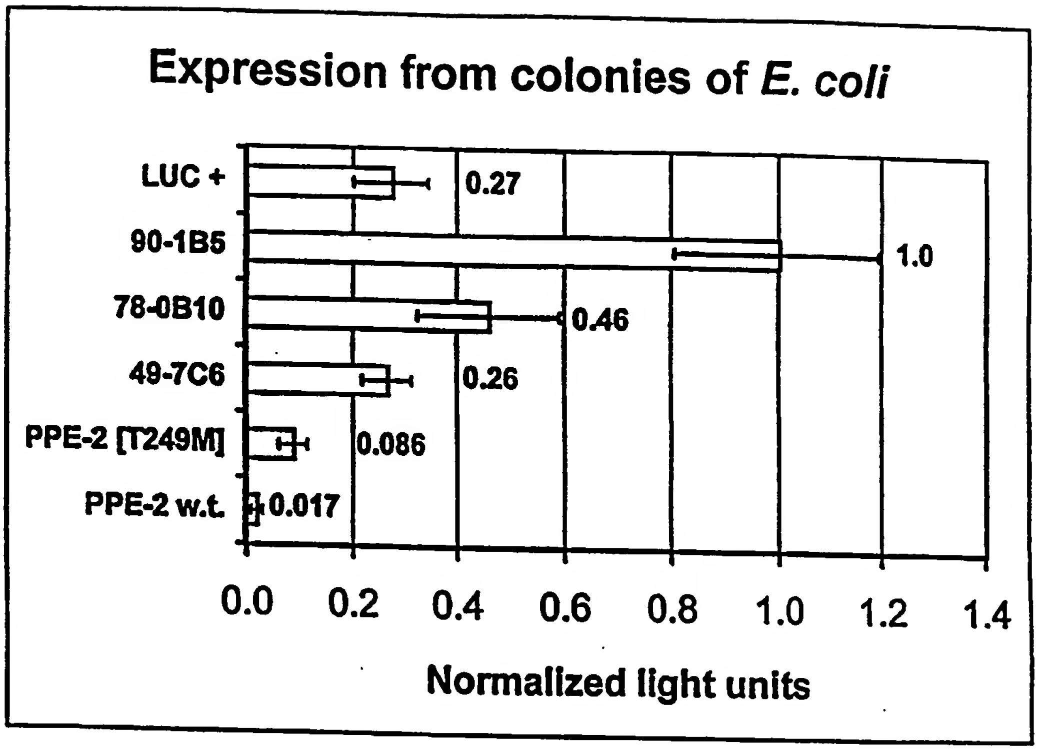

Expression from colonies of E. coli
LUC +
90-1B5
78-0B10
49-7C6
PPE-2 [T249M]
PPE-2 w.t.
0.27
1.0
0.46
0.26
0.086
0.017
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Normalized light units

FIG. 21

luc49-7C6 (SEQ ID NO:1)

```
GGATCCAATG GCAGATAAAA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGA AAATGTTTTA TATGAAGAGT TTTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTATA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TGGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCTTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAACAATG ACGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                             1639
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FIG. 22

luc49-6C10 (SEQ ID NO:2)

```
GGATCCAATG GAAGATAAAA ATATTTTATA TGGACCTGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TGTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTATA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TGGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCATGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTTTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAACAAATG ACGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AAACTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                             1639
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FIG. 23

luc49-0G12 (SEQ ID NO:3)

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GGATCCAATG GAAGATAAAA ATATTTTATA TGGACCTGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TTTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTATA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGTACGATT  660
TTCTTATGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAACAATG ACGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AAACTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                             1639
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FIG. 24

luc49-7A5 (SEQ ID NO:4)

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GGATCCAATG GAAGATAAAA ATATTTTATA TGGACCTGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TTTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTATA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTATTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTTTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAACAATG ACGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AAACTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                             1639
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FIG. 25

luc49-4G11 (SEQ ID NO:5)

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GGATCCAATG GAAGATAAAA ATATTTTATA TGGACCTGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTGACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TGTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTATA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCATGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTTTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAACAATG ACGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AAACTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                              1639
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FIG. 26

luc49-7C6 (SEQ ID NO:14)

		M	A	D	K	N	I	L	Y	G	P	E	P	P	Y	P	L	A	D	20
G	T	A	G	E	Q	M	F	Y	A	L	S	R	Y	A	D	I	S	G	C	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80
V	C	S	E	N	G	L	Q	F	F	L	P	I	I	A	S	L	Y	L	G	100
I	I	A	A	P	V	S	D	K	Y	I	E	R	E	L	I	H	S	L	G	120
I	V	K	P	R	I	I	F	C	S	K	N	T	F	Q	K	V	L	N	V	140
K	S	K	L	K	Y	V	E	T	I	I	I	L	D	L	N	E	D	L	G	160
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	I	N	L	D	V	K	180
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	L	V	M	F	S	S	200
G	T	T	G	V	S	K	G	V	M	L	T	H	K	N	I	V	A	R	F	220
S	L	A	K	D	P	T	F	G	N	A	I	N	P	T	T	A	I	L	T	240
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	260
F	R	V	V	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	280
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	300
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	320
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	340
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	N	N	D	V	R	P	360
G	S	T	G	K	I	V	P	F	H	A	V	K	V	V	D	P	T	T	G	380
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	D	M	I	M	K	400
G	Y	Y	N	N	E	E	A	T	K	A	I	I	N	K	D	G	W	L	R	420
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	440
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	L	460
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	E	480
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	Q	500
N	F	V	S	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	520
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	F	540
E	K	H	T	N	G															546

FIG. 27

Luc49-6C10 (SEQ ID NO:15)

```
    M E D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F Y A L S R Y A D I S G C  40
I A L T N A H T K E N V L Y E E L L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P I I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P Y S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V A R F 220
S H A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F F A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P N N D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I N K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G                            546
```

FIG. 28

Luc49-0G12 (SEQ ID NO:16)

```
  M E D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F Y A L S R Y A D I S G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P I I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P Y S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V V R F 220
S L A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F F A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P N N D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I T K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G                            546
```

FIG. 29

Luc49-7A5 (SEQ ID NO:17)

```
  M E D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F Y A L S R Y A D I S G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P I I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P Y S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V A R F 220
S I A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F L A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P N N D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I N K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G                            546
```

FIG. 30

Luc49-4G11 (SEQ ID NO:18)

```
  M E D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F D A L S R Y A D I S G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P I I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P Y S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V A R F 220
S H A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F F A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P N N D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I N K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G                            546
```

FIG. 31

luc78-0B10 (SEQ ID NO:6)

```
GGATCCAATG GCAGATAAGA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTGACGC ATTATCTCGT TATGCAGATA TTTCCGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGA AAATGTTTTA TATGAAGAGT TTTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATCTG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATAGTAATC TGGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCTTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCCACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAGGTG ACGCCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCGCCA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAATGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AGATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                               1639
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FIG. 32

luc78-0G8 (SEQ ID NO:7)

GGATCCAATG GCAGATAAAA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGCTGA 60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG 120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TTTTAAAATT 180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC 240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG 300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG 360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT 420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG 480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA 540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC 600
TGGTACAACT GGTGTTCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT 660
TTCTCTTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC 720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG 780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA 840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC 900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT 960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAxxxx xxGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGGGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG 1639

FIG. 33

luc78-1B1 (SEQ ID NO:8)

```
GGATCCAATG GCAGATAAAA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TGTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATATTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTATTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAxxxx xxGCCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCGCCA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                             1639
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FIG. 34

luc78-2B4 (SEQ ID NO:9)

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GGATCCAATG GCAGATAAAA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTGACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TGTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CGTTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATAGTAATC TGGACGTAAA  540
AAAATTTAAA CCAAATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCTTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAACxxxx xxGCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCGCCA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                            1639
```

FIG. 35

luc78-0B10 (SEQ ID NO:19)

		M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	A	D	20
G	T	A	G	E	Q	M	F	D	A	L	S	R	Y	A	D	I	S	G	C	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80
V	C	S	E	N	G	L	Q	F	F	L	P	V	I	A	S	L	Y	L	G	100
I	I	A	A	P	V	S	D	K	Y	I	E	R	E	L	I	H	S	L	G	120
I	V	K	P	R	I	I	F	C	S	K	N	T	F	Q	K	V	L	N	V	140
K	S	K	L	K	S	V	E	T	I	I	I	L	D	L	N	E	D	L	G	160
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	S	N	L	D	V	K	180
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	L	V	M	F	S	S	200
G	T	T	G	V	P	K	G	V	M	L	T	H	K	N	I	V	A	R	F	220
S	L	A	K	D	P	T	F	G	N	A	I	N	P	T	T	A	I	L	T	240
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	260
F	R	V	V	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	280
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	300
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	320
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	340
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	K	G	D	A	R	P	360
G	S	T	G	K	I	V	P	F	H	A	V	K	V	V	D	P	T	T	G	380
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	A	M	I	M	K	400
G	Y	Y	N	N	E	E	A	T	K	A	I	I	D	N	D	G	W	L	R	420
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	440
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	L	460
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	E	480
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	Q	500
D	F	V	S	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	520
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	F	540
E	K	H	T	N	G															546

FIG. 36

Luc78-0G8 (SEQ ID NO:20)

M A D K N I L Y G P E P F Y P L A D 20

G T A G E Q M F Y A L S R Y A D I S G C 40

I A L T N A H T K E N V L Y E E F L K L 60

S C R L A E S F K K Y G L K Q N D T I A 80

V C S E N G L Q F F L P V I A S L Y L G 100

I I A A P V S D K Y I E R E L I H S L G 120

I V K P R I I F C S K N T F Q K V L N V 140

K S K L K Y V E T I I I L D L N E D L G 160

G Y Q C L N N F I S Q N S D I N L D V K 180

K F K P Y S F N R D D Q V A L V M F S S 200

G T T G V P K G V M L T H K N I V A R F 220

S L A K D P T F G N A I N P T T A I L T 240

V I P F H H G F G M M T T L G Y F T C G 260

F R V V L M H T F E E K L F L Q S L Q D 280

Y K V E S T L L V P T L M A F L A K S A 300

L V E K Y D L S H L K E I A S G G A P L 320

S K E I G E M V K K R F K L N F V R Q G 340

Y G L T E T T S A V L I T P K X X V R P 360

G S T G K I V P F H A V K V V D P T T G 380

K I L G P N E P G E L Y F K G D M I M K 400

G Y Y N N E E A T K A I I D K D G W L R 420

S G D I A Y Y D N D G H F Y I V D R L K 440

S L I K Y K G Y Q V A P A E I E G I L L 460

Q H P Y I V D A G V T G I P D E A A G E 480

L P A A G V V V Q T G K Y L N E Q I V Q 500

N F V S S Q V S T A K W L R G G V K F L 520

D E I P K G S T G K I D R K V L R Q M F 540

E K H T N G 546

FIG. 37

Luc78-1E1 (SEQ ID NO:21)

M A D K N I L Y G P E P F Y P L A D 20
G T A G E Q M F D A L S R Y A D I P G C 40
I A L T N A H T K E N V L Y E E F L K L 60
S C R L A E S F K K Y G L K Q N D T I A 80
V C S E N G L Q Y F L P V I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P N S F N R D D Q V A L V M F S S 200
G T T G V P K G V M L T H K N I V A R F 220
S I A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F L A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P K X X A R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E P G E L Y F K G A M I M K 400
G Y Y N N E E A T K A I I D K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G 546

FIG. 38

Luc78-2B4 (SEQ ID NO:22)

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  M A D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F D A L S R Y A D I P G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P V I A S L Y L G 100
I I A A P V S D K Y V E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D S N L D V K 180
K F K P N S F N R D D Q V A L V M F S S 200
G T T G V P K G V M L T H K N I V A R F 220
S L A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F L A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P K X X A R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G A M I M K 400
G Y Y N N E E A T K A I I D K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G                            546
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FIG. 39

luc85-4F12 (SEQ ID NO:10)

GGATCCAATG GCAGATAAAA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGCTGA 60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTCCGGGCTG 120
CATAGCATTG ACAAATGCTC ATACAAAAGC CCCTGTTTTA TATGAAGAGT TTTTAAAATT 180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC 240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG 300
AATAATTGTG GCACCTGTTA ACGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG 360
TATTGTAAAA CCACGCATAG TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT 420
AAAATCTAAA TTAAAATCTG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG 480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA 540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGATTA TGTTTTCTTC 600
TGGTACAACT GGTCTGCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT 660
TTCTCTTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCCACGACAG CAATTTTAAC 720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG 780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA 840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC 900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT 960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAxxxx xxGCCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCCCGA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAATGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATT CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG 1639

FIG. 40

Luc85-4F12 (SEQ ID NO:23)

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    M A D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F D A L S R Y A D I P G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P V I A S L Y L G 100
I I V A P V N D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K S V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P Y S F N R D D Q V A L I M F S S 200
G T T G L P K G V M L T H K N I V A R F 220
S L A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F L A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P K X X A R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E P G E L Y F K G P M I M K 400
G Y Y N N E E A T K A I I D N D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
D F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G                             546
```

FIG. 41

Luc90-1B5 (SEQ ID NO:11)

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GGATCCAATG GCAGATAAGA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGAAGA   60
TGGGACGGCT GGAGAACAGA TGTTTGACGC ATTATCTCGT TATGCAGATA TTCCGGGCTG  120
CATAGCATTG ACAAATGCTC ATACAAAAGA AAATGTTTTA TATGAAGAGT TTCTGAAACT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTC TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG  300
AATAATTGTG GCACCTGTTA ACGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAG TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATCTA TTGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATAGTAATC TGGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGATTA TGTTTTCTTC  600
TGGTACAACT GGTCTGCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCTTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCCACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAGGTG ACGCCAAACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCCCGA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAATGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCACTGATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AGATTATGTT GCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC ACCAATGGG                                             1639
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FIG. 42

Luc90-1B5 (SEQ ID NO:24)

		M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	E	D	20
G	T	A	G	E	Q	M	F	D	A	L	S	R	Y	A	D	I	P	G	C	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80
V	C	S	E	N	G	L	Q	F	F	L	P	V	I	A	S	L	Y	L	G	100
I	I	V	A	P	V	N	D	K	Y	I	E	R	E	L	I	H	S	L	G	120
I	V	K	P	R	I	V	F	C	S	K	N	T	F	Q	K	V	L	N	V	140
K	S	K	L	K	S	I	E	T	I	I	I	L	D	L	N	E	D	L	G	160
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	S	N	L	D	V	K	180
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	L	I	M	F	S	S	200
G	T	T	G	L	P	K	G	V	M	L	T	H	K	N	I	V	A	R	F	220
S	L	A	K	D	P	T	F	G	N	A	I	N	P	T	T	A	I	L	T	240
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	260
F	R	V	V	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	280
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	300
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	320
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	340
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	K	G	D	A	K	P	360
G	S	T	G	K	I	V	P	F	H	A	V	K	V	V	D	P	T	T	G	380
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	P	M	I	M	K	400
G	Y	Y	N	N	E	E	A	T	K	A	I	I	D	N	D	G	W	L	R	420
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	440
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	L	460
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	E	480
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	Q	500
D	Y	V	A	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	520
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	F	540
E	K	H	T	N	G															546

FIG. 43

lucPpe2 [T249M] (SEQ ID NO:12)

```
GGATCCAATG GAAGATAAAA ATATTTTATA TGGACCTGAA CCATTTTATC CCTTGGCTGA   60
TGGGACGGCT GGAGAACAGA TGTTTTACGC ATTATCTCGT TATGCAGATA TTTCAGGATG  120
CATAGCATTG ACAAATGCTC ATACAAAAGA AAATGTTTTA TATGAAGAGT TTTTAAAATT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATGGTT TGCAATTTTT CCTTCCTTTA ATTGCATCAT TGTATCTTGG  300
AATAATTGCA GCACCTGTTA GTGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAA TTTTTTGTTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATATG TAGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATATTAATC TTGACGTAAA  540
AAAATTTAAA CCAAATTCTT TTAATCGAGA CGATCAGGTT GCGTTGGTAA TGTTTTCTTC  600
TGGTACAACT GGTGTTTCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTCATTGC AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCAACGACAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GCTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTTTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGGACACTG ACGTCAGACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AAACTGGAGA ATTGTATTTT AAAGGCGACA TGATAATGAA 1200
AAGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT AACAAAGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCATTAATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AAATTTTGTT TCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
TGAAAAACAC AAATCTAAGC TG                                          1642
```

FIG. 44

LucPpe2 [T249M] (SEQ ID NO:25)

```
   M E D K N I L Y G P E P F Y P L A D  20
G T A G E Q M F Y A L S R Y A D I S G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N G L Q F F L P L I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P N S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V A R F 220
S H C K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V A L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F F A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P D T D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I N K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H K S K L                           547
```

FIG. 45

Luc*PpL* 81-6G1 (SEQ ID NO:26)

```
M M K R E K N V I Y G P E P L H P L E D  20
L T A G E M L F R A L R K H S H L P Q A  40
L V D V V G D E S L S Y K E F F E A T V  60
L L A Q S L H N C G Y K M N D V V S I C  80
A E N N T R F F I P V I A A W Y I G M I 100
V A P V N E S Y I P D E L C K V M G I S 120
K P Q I V F T T K N I L N K V L E V Q S 140
R T N F I K R I I I L D T V E N I H G C 160
E S L P N G I S R Y S D G N I A N F K P 180
L H F D P V E Q V A A I L C S S G T T G 200
L P K G V M Q T H Q N I C V R L I H A L 220
D P R A G T Q L I P G V T V L V Y L P F 240
F H A F G F S I T L G Y F M V G L R V I 260
M F R R F D Q E A F L K A I Q D Y E V R 280
S V I N V P S V I L F L S K S P L V D K 300
Y D L S S L R E L C C G A A P L A K E V 320
A E V A A K R L N L P G I R C G F G L T 340
E S T S A N I H S L R D E F K S G S L G 360
R V T P L M A A K I A D R E T G K A L G 380
P N Q V G E L C I K G P M V S K G Y V N 400
N V E A T K E A I D D D G W L H S G D F 420
G Y Y D E D E H F Y V V D R Y K E L I K 440
Y K G S Q V A P A E L E E I L L K N P C 460
I R D V A V V G I P D L E A G E L P S A 480
F V V K Q P G K E I T A K E V Y D Y L A 500
E R V S H T K Y L R G G V R F V D S I P 520
R N V T G K I T R K E L L K Q L L E K A 540
G G                                     542
```

FIG. 46

Luc*Ppl* 81-6G1

ATGATGAAGC GAGAGAAAAA TGTTATATAT GGACCCGAAC CCCTACACCC CTTGGAAGAC
TTAACAGCTG GAGAAATGCT CTTCCGTGCC CTTCGAAAAC ATTCTCATTT ACCGCAGGCT
TTAGTAGATG TGGTTGGCGA CGAATCGCTT TCCTATAAAG AGTTTTTTGA AGCGACAGTC
CTCCTAGCGC AAAGTCTCCA CAATTGTGGA TACAAGATGA ATGATGTAGT GTCGATCTGC
GCCGAGAATA ATACAAGATT TTTTATTCCC GTTATTGCAG CTTGGTATAT TGGTATGATT
GTAGCACCTG TTAATGAAAG TTACATCCCA GATGAACTCT GTAAGGTGAT GGGTATATCG
AAACCACAAA TAGTTTTTAC GACAAAGAAC ATTTTAAATA AGGTATTGGA GGTACAGAGC
AGAACTAATT TCATAAAAAG GATCATCATA CTTGATACTG TAGAAAACAT ACACGGTTGT
GAAAGTCTTC CCAATTTTAT TTCTCGTTAT TCGGATGGAA ATATTGCCAA CTTCAAACCT
TTACATTTCG ATCCTGTTGA GCAAGTGGCA GCTATCTTAT GTTCGTCAGG CACTACTGGA
TTACCGAAAG GTGTAATGCA AACTCACCAA AATATTTGTG TCCGACTTAT ACATGCTTTA
GACCCCAGGG CAGGAACGCA ACTTATTCCT GGTGTGACAG TCTTAGTATA TCTGCCTTTT
TTCCATGCTT TTGGGTTCTC TATAACCTTG GGATACTTCA TGGTGGGTCT TCGTGTTATC
ATGTTCAGAC GATTTGATCA AGAAGCATTT CTAAAAGCTA TTCAGGATTA TGAAGTTCGA
AGTGTAATTA ACGTTCCATC AGTAATATTG TTCTTATCGA AAAGTCCTTT GGTTGACAAA
TACGATTTAT CAAGTTTAAG GGAATTGTGT TGCGGTGCGG CACCATTAGC AAAAGAAGTT
GCTGAGGTTG CAGCAAAACG ATTAAACTTG CCAGGAATTC GCTGTGGATT TGGTTTGACA
GAATCTACTT CAGCTAATAT ACACAGTCTT AGGGATGAAT TTAAATCAGG ATCACTTGGA
AGAGTTACTC CTTTAATGGC AGCTAAAATA GCAGATAGGG AAACTGGTAA AGCATTGGGA
CCAAATCAAG TTGGTGAATT ATGCATTAAA GGTCCCATGG TATCGAAAGG TTACGTGAAC
AATGTAGAAG CTACCAAAGA AGCTATTGAT GATGATGGTT GGCTTCACTC TGGAGACTTT
GGATACTATG ATGAGGATGA GCATTTCTAT GTGGTGGACC GTTACAAGGA ATTGATTAAA
TATAAGGGCT CTCAGGTAGC ACCTGCAGAA CTAGAAGAGA TTTTATTGAA AAATCCATGT
ATCAGAGATG TTGCTGTGGT TGGTATTCCT GATCTAGAAG CTGGAGAACT GCCATCTGCG
TTTGTGGTTA AACAGCCCGG AAAGGAGATT ACAGCTAAAG AAGTGTACGA TTATCTTGCC
GAGAGGGTCT CCCATACAAA GTATTTGCGT GGAGGGGTTC GATTCGTTGA TAGCATACCA
AGGAATGTTA CAGGTAAAAT TACAAGAAAG GAACTTCTGA AGCAGTTGCT GGAGAAGGCG
GGAGGT

FIG. 47

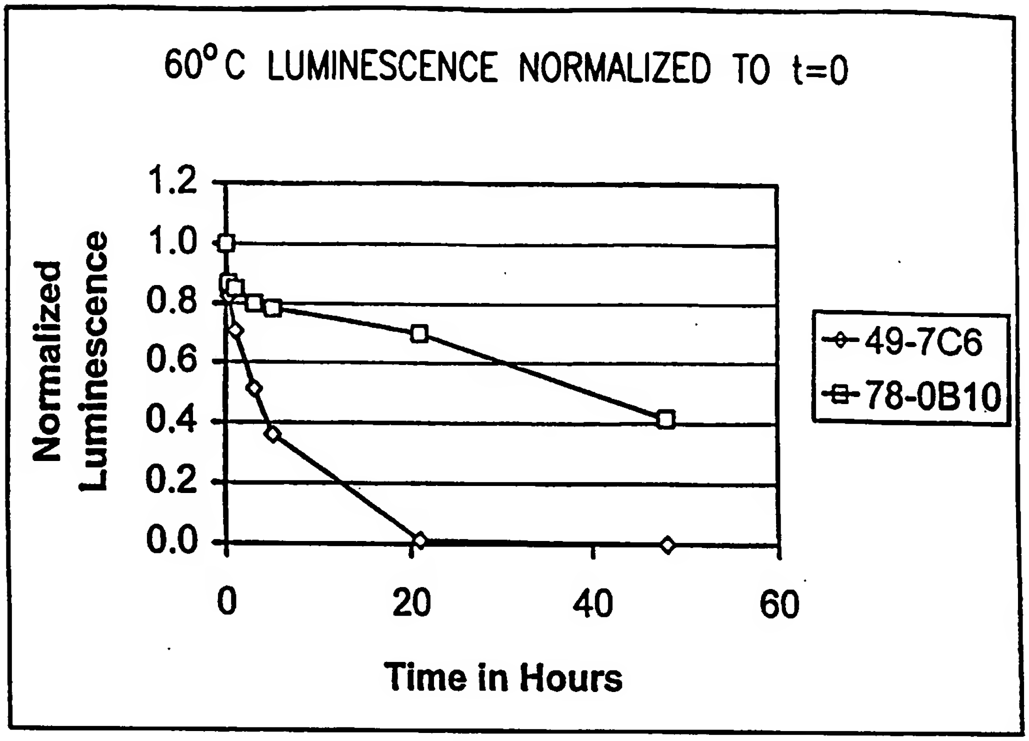
60° C LUMINESCENCE NORMALIZED TO t=0
Normalized Luminescence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
Time in Hours
49-7C6
78-0B10

FIG. 48

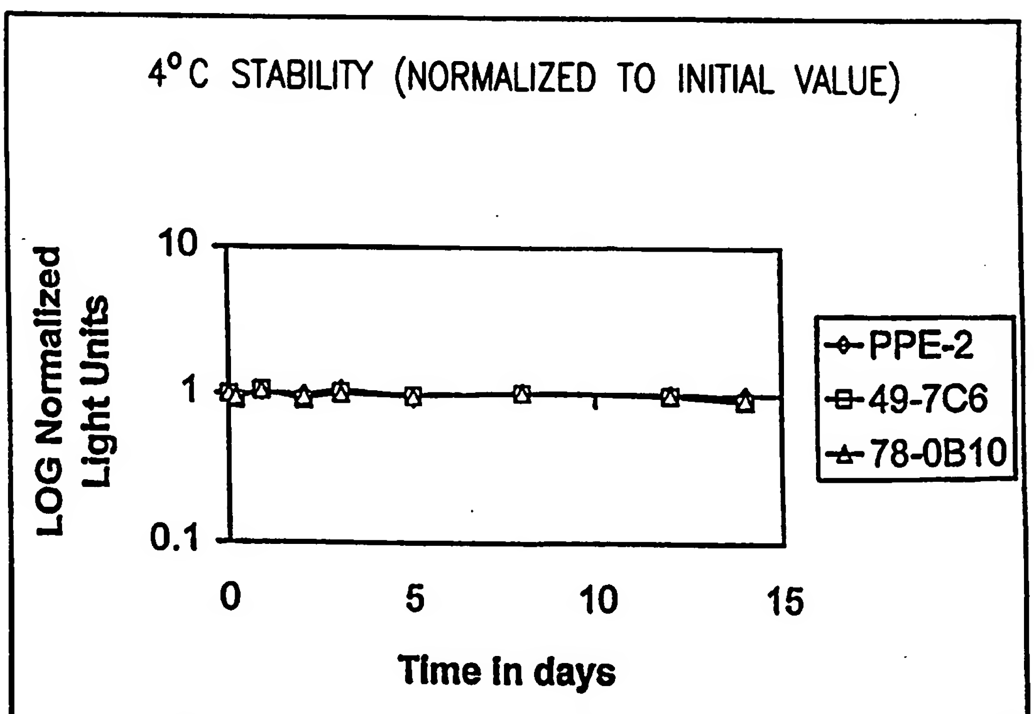
4° C STABILITY (NORMALIZED TO INITIAL VALUE)
LOG Normalized Light Units
10
1
0.1
0
5
10
15
Time in days
PPE-2
49-7C6
78-0B10

FIG. 49

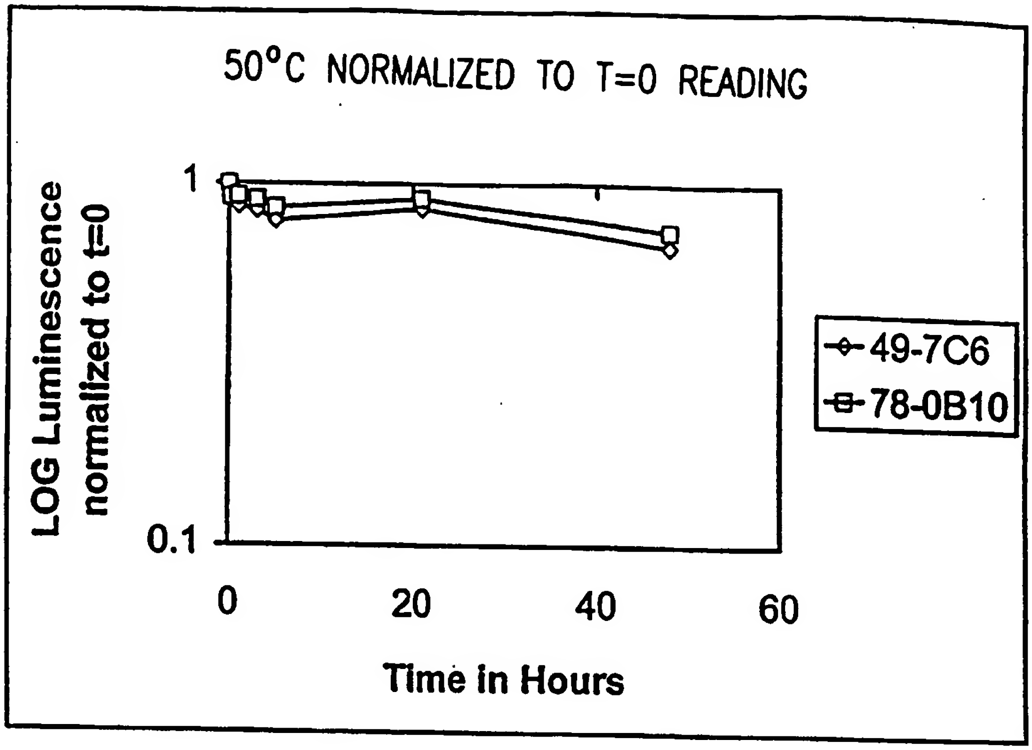
50°C NORMALIZED TO T=0 READING
LOG Luminescence normalized to t=0
1
0.1
0
20
40
60
Time in Hours
49-7C6
78-0B10

FIG. 50

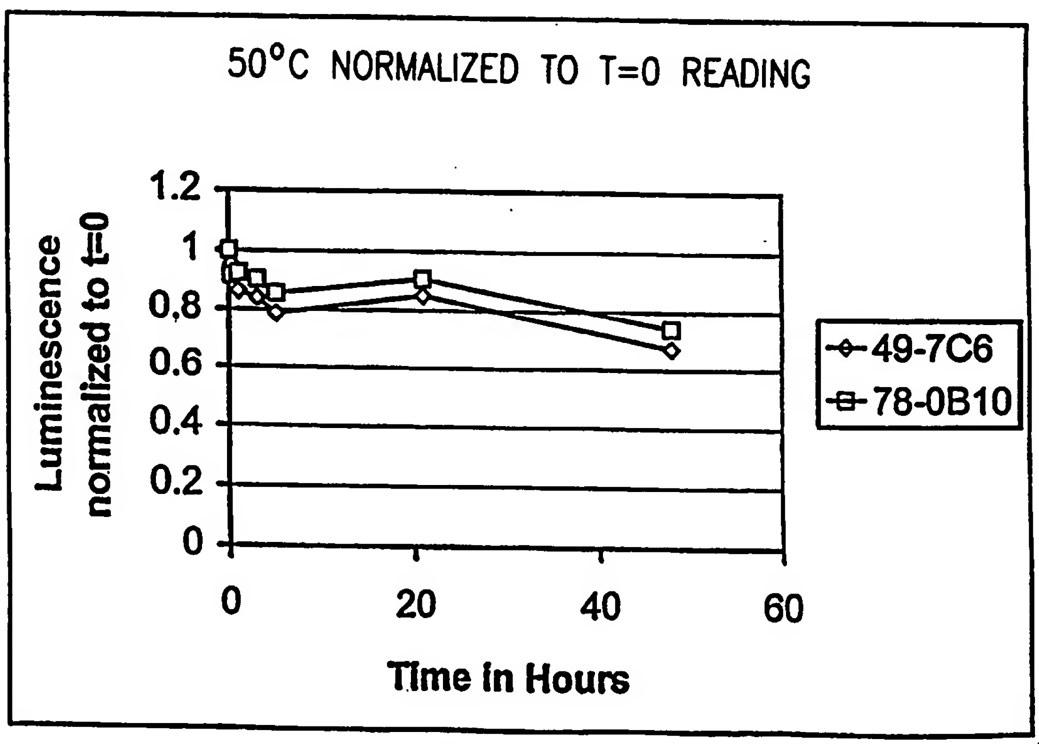
50°C NORMALIZED TO T=0 READING
Luminescence normalized to t=0
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
Time in Hours
49-7C6
78-0B10

FIG. 51

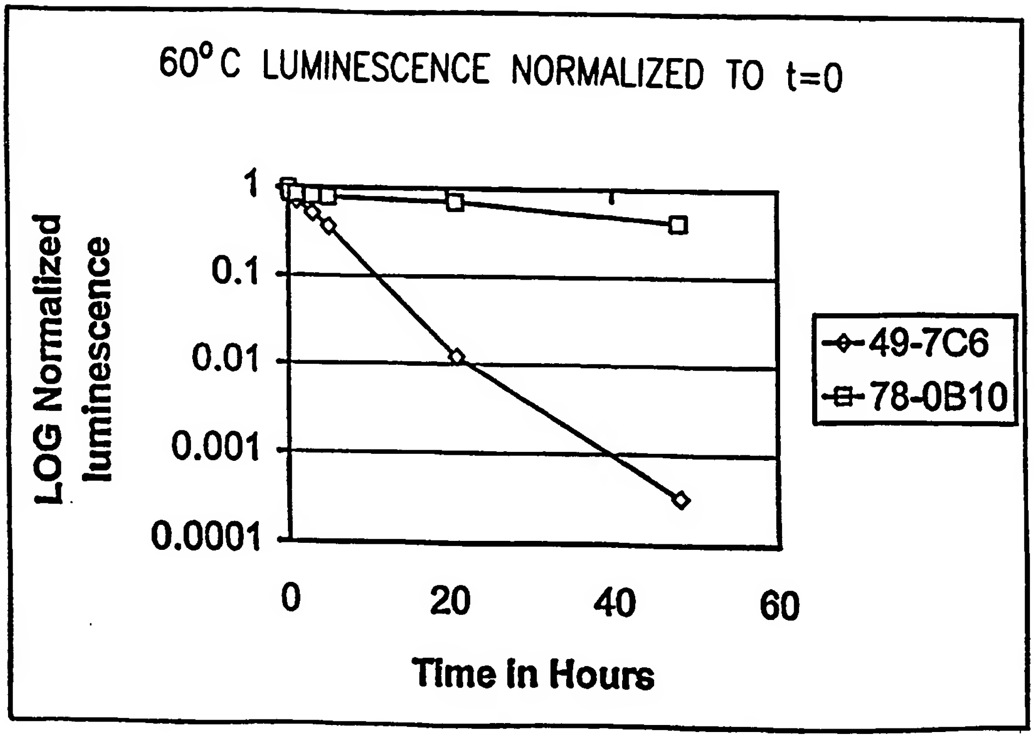
60° C LUMINESCENCE NORMALIZED TO t=0
LOG Normalized luminescence
1
0.1
0.01
0.001
0.0001
0
20
40
60
Time in Hours
49-7C6
78-0B10

FIG. 52

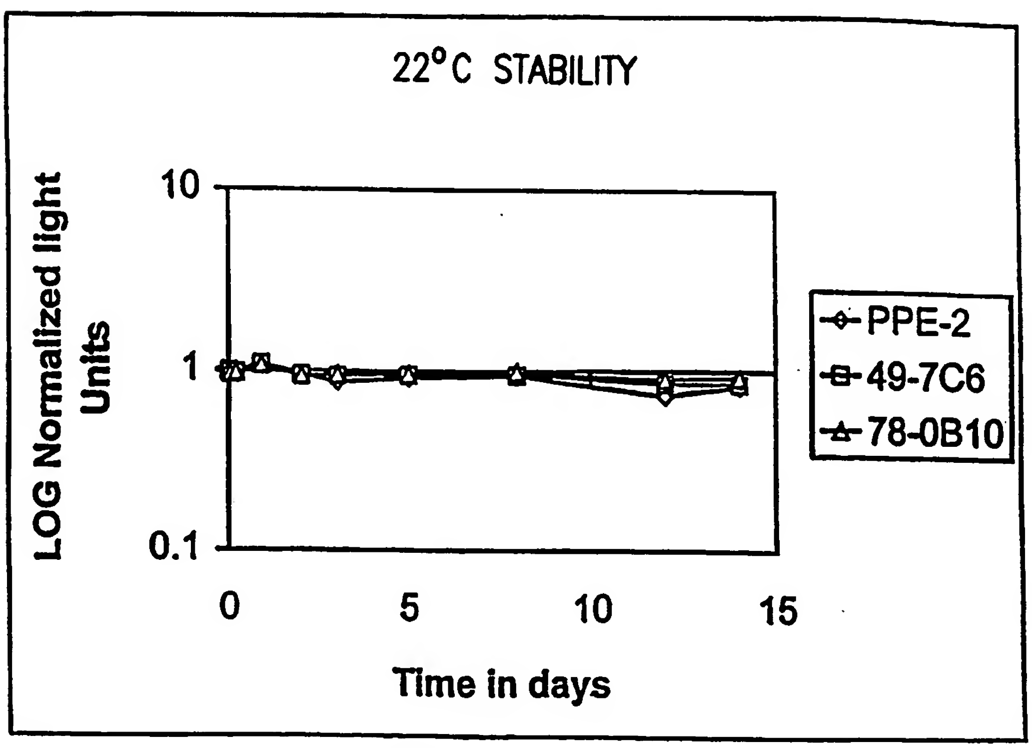
22° C STABILITY
LOG Normalized light Units
10
1
0.1
0
5
10
15
Time in days
PPE-2
49-7C6
78-0B10

FIG. 53

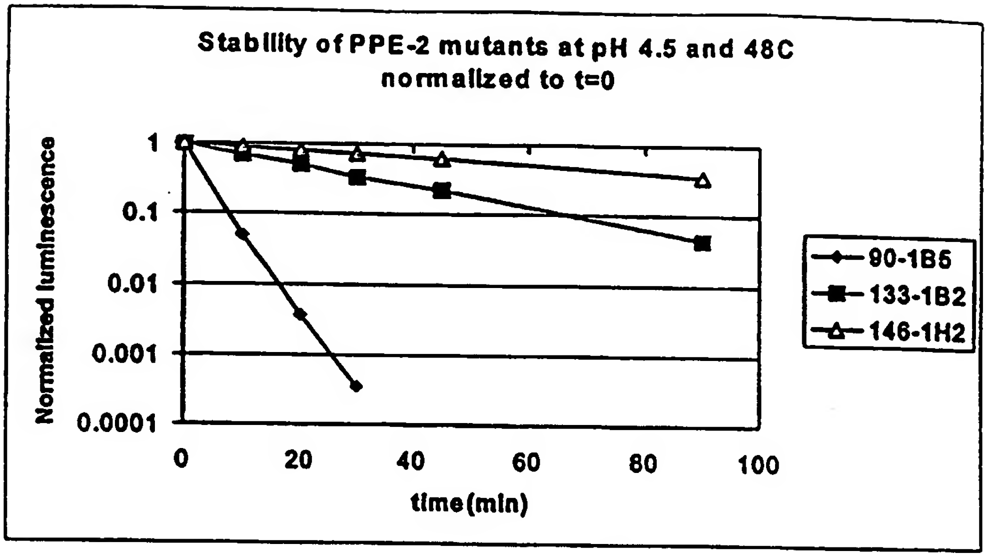

FIG. 54A

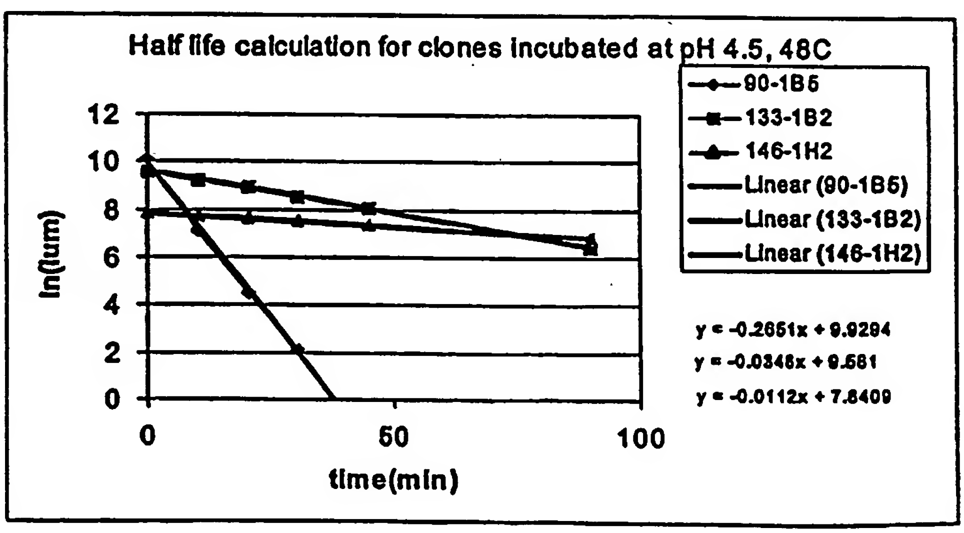

FIG. 54B

Luc133-1B2 (SEQ ID NO:42)

```
AGATCCAATG GCAGATAAGA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGAAGA   60
TGGGACGGCT GGAGAACAGA TGTTTGACGC ATTATCTCGT TATGCAGATA TTCCGGGCTG  120
CATAGCATTG ACAAATGCTC ATACAAAAGA AAATGTTTTA TATGAAGAGT TTCTGAAACT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATAGTC TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG  300
AATAATTGTG GCACCTGTTA ACGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAG TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATCTA TTGAAACTAT TATTATATTA GACTTAAATG ATGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATAGTAATC TGGACGTAAA  540
AAAATTTAAA CCATATTCTT TTAATCGAGA CGATCAGGTT GCGTTGATTA TGTTTTCTTC  600
TGGTACAACT GGTCTGCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTATTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCCACGTCAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAGGTG ACGCCAAACC 1080
GGGATCAACT GGTAAAATAG TACCATTTCA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCCCGA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAATGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCACTGATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AGATTATGTT GCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGATATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
AGAAAAACAC ACCAATGGG                                               1639
```

FIG. 55

Luc146-1H2 (SEQ ID NO:43)

```
GGATCCAATG GCAGATAAGA ATATTTTATA TGGGCCCGAA CCATTTTATC CCTTGGAAGA   60
TGGGACGGCT GGAGAACAGA TGTTTGACGC ATTATCTCGT TATGCAGCTA TTCCGGGCTG  120
CATAGCATTG ACAAATGCTC ATACAAAAGA AAATGTTTTA TATGAAGAGT TTCTGAAACT  180
GTCGTGTCGT TTAGCGGAAA GTTTTAAAAA GTATGGATTA AAACAAAACG ACACAATAGC  240
GGTGTGTAGC GAAAATAGTC TGCAATTTTT CCTTCCTGTA ATTGCATCAT TGTATCTTGG  300
AATAATTGTG GCACCTGTTA ACGATAAATA CATTGAACGT GAATTAATAC ACAGTCTTGG  360
TATTGTAAAA CCACGCATAG TTTTTTGCTC CAAGAATACT TTTCAAAAAG TACTGAATGT  420
AAAATCTAAA TTAAAATCTA TTGAAACTAT TATTATATTA GACTTAAATG AAGACTTAGG  480
AGGTTATCAA TGCCTCAACA ACTTTATTTC TCAAAATTCC GATAGTAATC TGGACGTAAA  540
AAAATTTAAA CCCTATTCTT TTAATCGAGA CGATCAGGTT GCGTCGATTA TGTTTTCTTC  600
TGGTACAACT GGTCTGCCGA AGGGAGTCAT GCTAACTCAC AAGAATATTG TTGCACGATT  660
TTCTATTGCA AAAGATCCTA CTTTTGGTAA CGCAATTAAT CCCACGTCAG CAATTTTAAC  720
GGTAATACCT TTCCACCATG GTTTTGGTAT GATGACCACA TTAGGATACT TTACTTGTGG  780
ATTCCGAGTT GTTCTAATGC ACACGTTTGA AGAAAAACTA TTTCTACAAT CATTACAAGA  840
TTATAAAGTG GAAAGTACTT TACTTGTACC AACATTAATG GCATTTCTTG CAAAAAGTGC  900
ATTAGTTGAA AAGTACGATT TATCGCACTT AAAAGAAATT GCATCTGGTG GCGCACCTTT  960
ATCAAAAGAA ATTGGGGAGA TGGTGAAAAA ACGGTTTAAA TTAAACTTTG TCAGGCAAGG 1020
GTATGGATTA ACAGAAACCA CTTCGGCTGT TTTAATTACA CCGAAAGGTG ACGCCAAACC 1080
GGGATCAACT GGTAAAATAG TACCATTACA CGCTGTTAAA GTTGTCGATC CTACAACAGG 1140
AAAAATTTTG GGGCCAAATG AACCTGGAGA ATTGTATTTT AAAGGCCCGA TGATAATGAA 1200
GGGTTATTAT AATAATGAAG AAGCTACTAA AGCAATTATT GATAATGACG GATGGTTGCG 1260
CTCTGGTGAT ATTGCTTATT ATGACAATGA TGGCCATTTT TATATTGTGG ACAGGCTGAA 1320
GTCACTGATT AAATATAAAG GTTATCAGGT TGCACCTGCT GAAATTGAGG GAATACTCTT 1380
ACAACATCCG TATATTGTTG ATGCCGGCGT TACTGGTATA CCGGATGAAG CCGCGGGCGA 1440
GCTTCCAGCT GCAGGTGTTG TAGTACAGAC TGGAAAATAT CTAAACGAAC AAATCGTACA 1500
AGATTATGTT GCCAGTCAAG TTTCAACAGC CAAATGGCTA CGTGGTGGGG TGAAATTTTT 1560
GGATGAAATT CCCAAAGGAT CAACTGGAAA AATTGACAGA AAAGTGTTAA GACAAATGTT 1620
AGAAAAACAC ACCAATGGG                                             1639
```

FIG. 56

Luc133-1B2 (SEQ ID NO:44)

```
    M A D K N I L Y G P E P F Y P L E D  20
G T A G E Q M F D A L S R Y A D I P G C  40
I A L T N A H T K E N V L Y E E F L K L  60
S C R L A E S F K K Y G L K Q N D T I A  80
V C S E N S L Q P F L P V I A S L Y L G 100
I I V A P V N D K Y I E R E L I H S L G 120
I V K P R I V F C S K N T F Q K V L N V 140
K S K L K S I E T I I I L D L N D D L G 160
G Y Q C L N N F I S Q N S D S N L D V K 180
K F K P Y S F N R D D Q V A L I M F S S 200
G T T G L P K G V M L T H K N I V A R F 220
S I A K D P T F G N A I N P T S A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F L A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P K G D A K P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E P G E L Y F K G P M I M K 400
G Y Y N N E E A T K A I I D N D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
D Y V A S Q V S T A K W L R G G V I F L 520
D E I P K G S T G K I D R K V L R Q M L 540
E K H T N G
```

FIG. 57

Luc146-1H2 (SEQ ID NO:45)

		M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	E	D	20
G	T	A	G	E	Q	M	F	D	A	L	S	R	Y	A	A	I	P	G	C	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80
V	C	S	E	N	S	L	Q	F	F	L	P	V	I	A	S	L	Y	L	G	100
I	I	V	A	P	V	N	D	K	Y	I	E	R	E	L	I	H	S	L	G	120
I	V	K	P	R	I	V	F	C	S	K	N	T	F	Q	K	V	L	N	V	140
K	S	K	L	K	S	I	E	T	I	I	I	L	D	L	N	E	D	L	G	160
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	S	N	L	D	V	K	180
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	S	I	M	F	S	S	200
G	T	T	G	L	P	K	G	V	M	L	T	H	K	N	I	V	A	R	F	220
S	I	A	K	D	P	T	F	G	N	A	I	N	P	T	S	A	I	L	T	240
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	260
F	R	V	V	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	280
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	300
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	320
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	340
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	K	G	D	A	K	P	360
G	S	T	G	K	I	V	P	L	H	A	V	K	V	V	D	P	T	T	G	380
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	P	M	I	M	K	400
G	Y	Y	N	N	E	E	A	T	K	A	I	I	D	N	D	G	W	L	R	420
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	440
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	L	460
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	E	480
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	Q	500
D	Y	V	A	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	520
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	L	540
E	K	H	T	N	G															546

FIG. 58

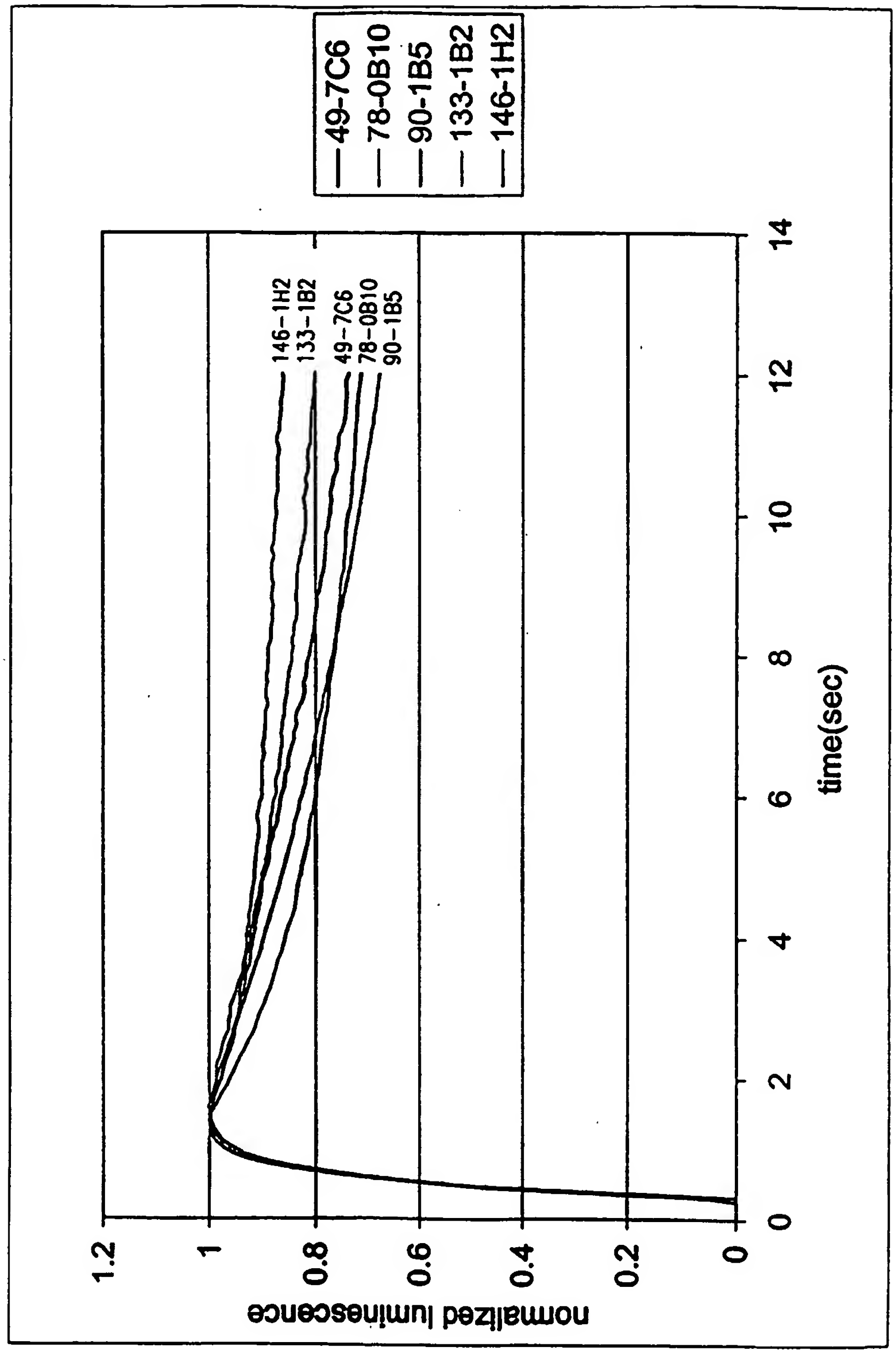
49-7C6
78-0B10
90-1B5
133-1B2
146-1H2
normalized luminescence
time(sec)
0
0.2
0.4
0.6
0.8
1
1.2
0
2
4
6
8
10
12
14

FIG. 59

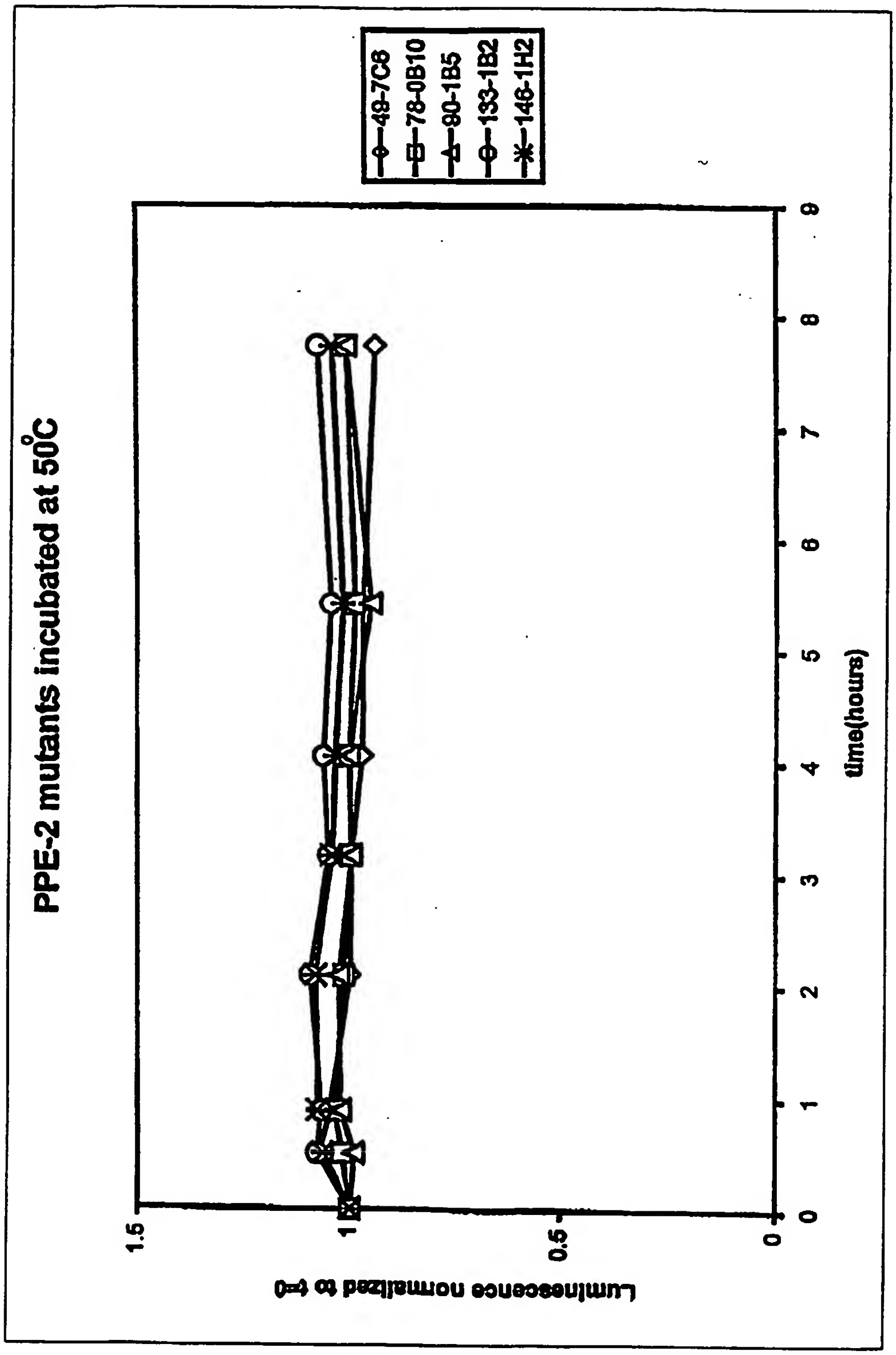
PPE-2 mutants incubated at 50°C
49-7C8
78-0B10
90-1B5
133-1B2
146-1H2
Luminescence normalized to t=0
1.5
1
0.5
0
0
1
2
3
4
5
6
7
8
9
time(hours)

FIG. 60

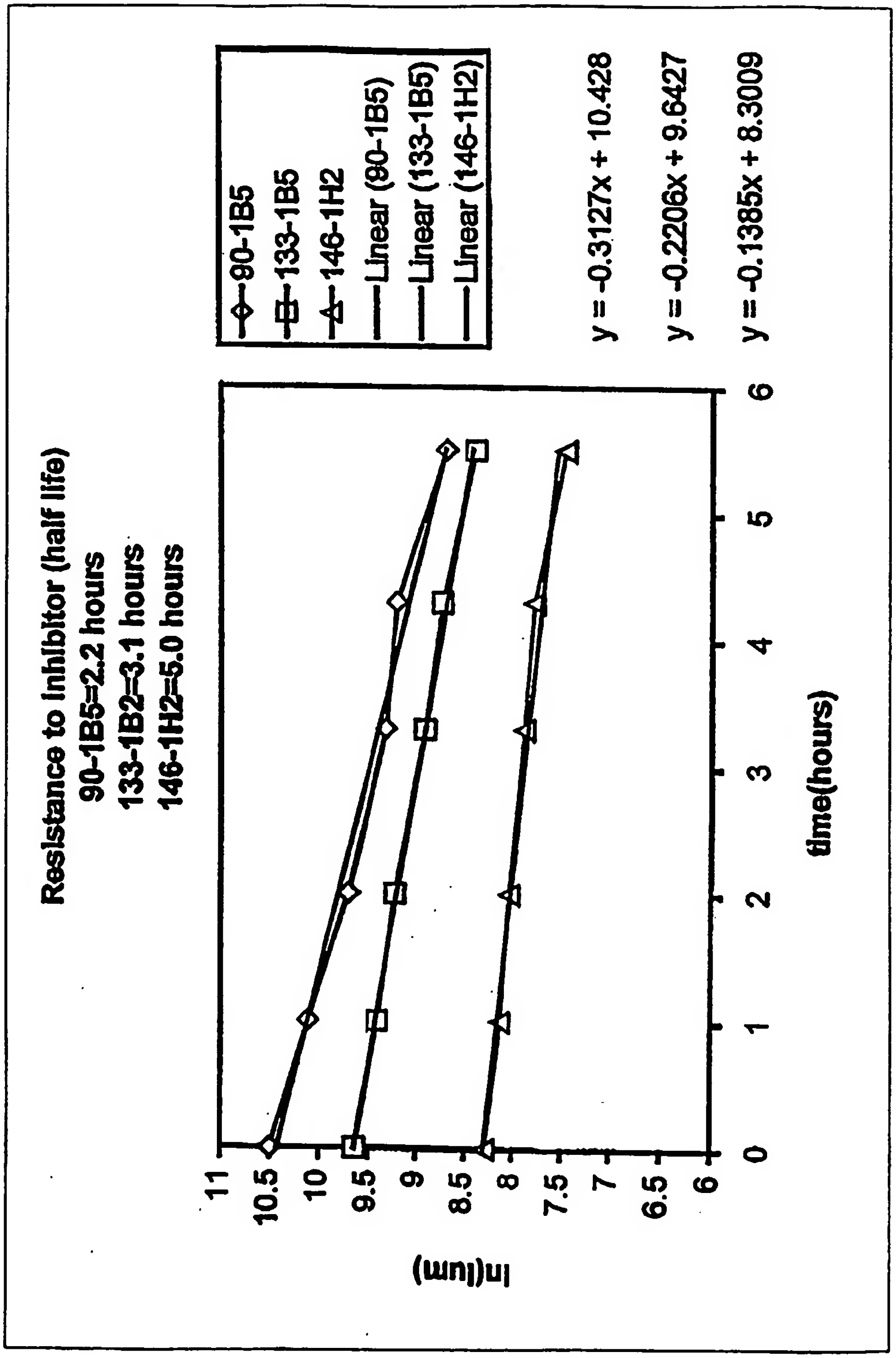
Resistance to Inhibitor (half life)
90-1B5=2.2 hours
133-1B2=3.1 hours
146-1H2=5.0 hours
90-1B5
133-1B5
146-1H2
Linear (90-1B5)
Linear (133-1B5)
Linear (146-1H2)
y = -0.3127x + 10.428
y = -0.2206x + 9.6427
y = -0.1385x + 8.3009
ln(lum)
11
10.5
10
9.5
9
8.5
8
7.5
7
6.5
6
0
1
2
3
4
5
6
time(hours)

FIG. 61

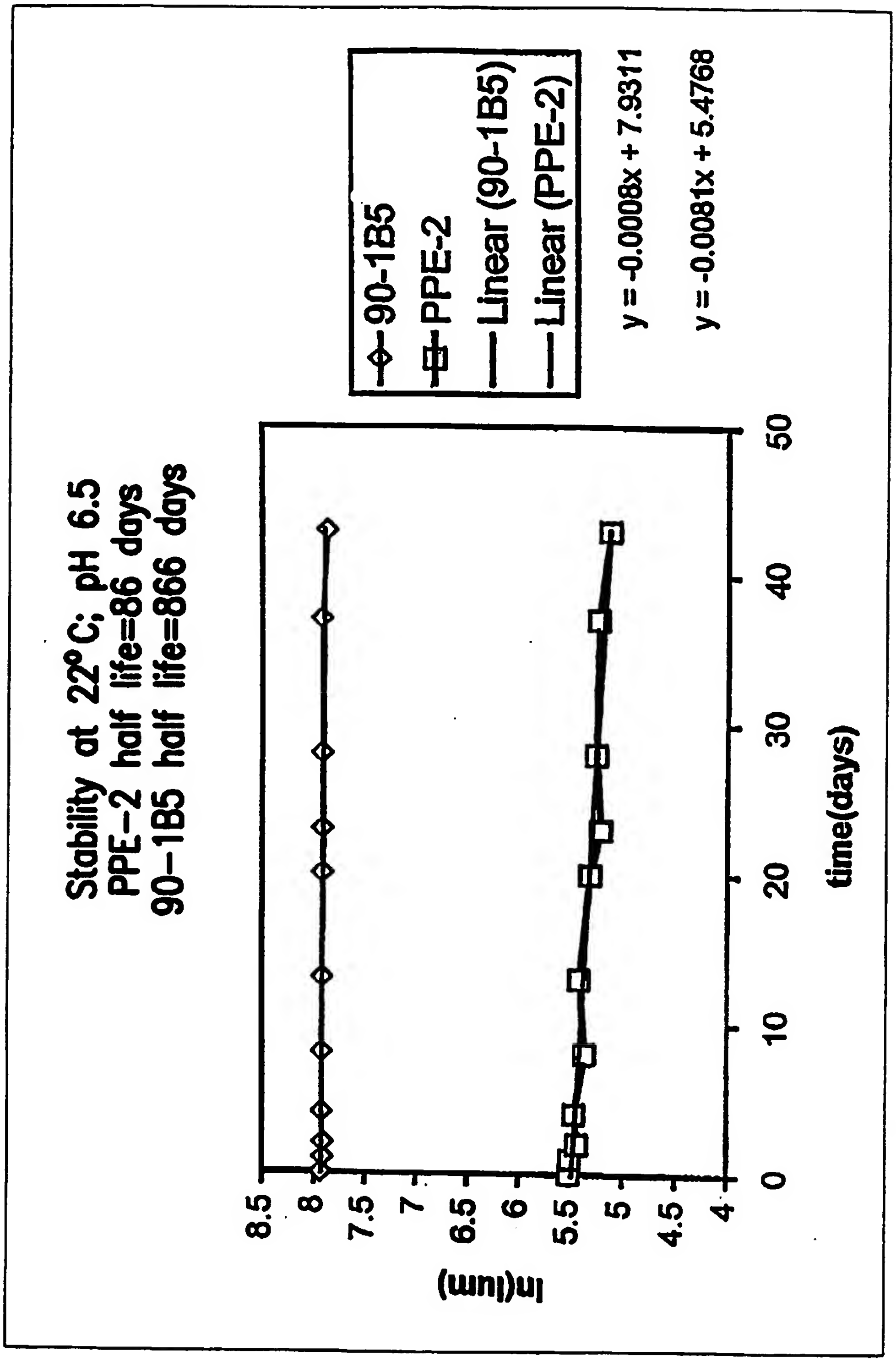
Stability at 22°C; pH 6.5
PPE–2 half life=86 days
90–1B5 half life=866 days
90-1B5
PPE-2
Linear (90-1B5)
Linear (PPE-2)
y = -0.0008x + 7.9311
y = -0.0081x + 5.4768
ln(lum)
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
0
10
20
30
40
50
time(days)

FIG. 62

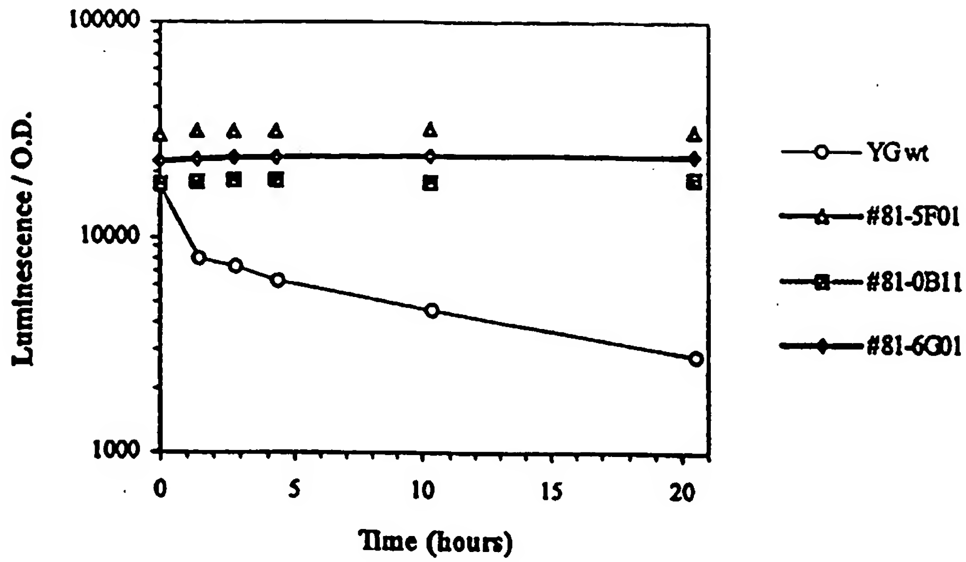
100000
10000
1000
Luminescence / O.D.
0
5
10
15
20
Time (hours)
YG wt
#81-5F01
#81-0B11
#81-6G01

FIG. 63

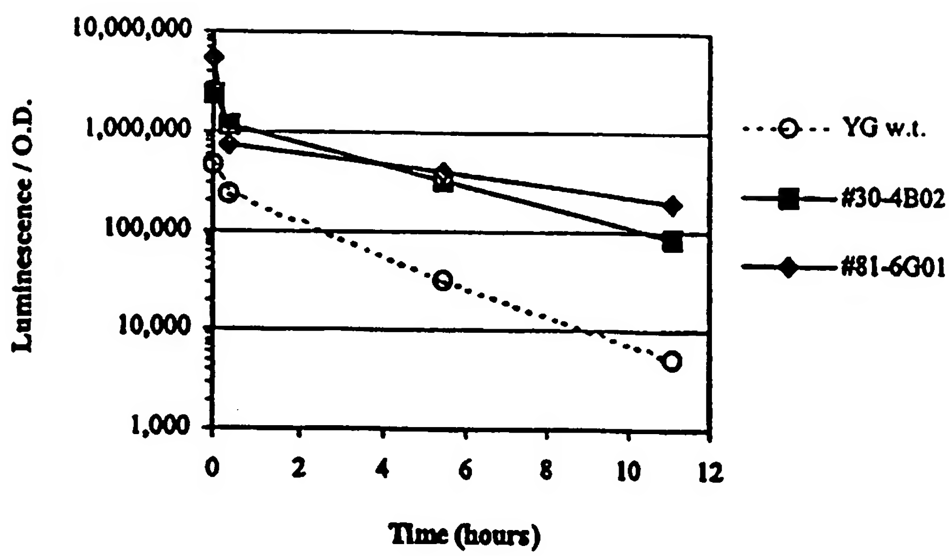
10,000,000
1,000,000
100,000
10,000
1,000
Luminescence / O.D.
0
2
4
6
8
10
12
Time (hours)
YG w.t.
#30-4B02
#81-6G01

FIG. 64

LucPpl81-0B11

```
GGATCCCATG ATGAAGCGAG AGAAAAATGT TATATATGGA CCCGAACCCC TACACCCCTT 60
GGAAGACTTA ACAGCTGGAG AAATGCTCTT CCGTGCCCTT CGAAAACATT CTCATTTACC 120
GCAGGCTTTA GTAGATGTGG TTGGCGACGA ATCGCTTTCC TATAAAGAGT TTTTTGAAGC 180
GACAGTCCTC CTAGCGCAAA GTCTCCACAA TTGTGGATAC AAGATGAATG ATGTAGTGTC 240
GATCTGCGCC GAGAATAATA CAAGATTTTT TATTCCCGTT ATTGCAGCTT GGTATATTGG 300
TATGATTGTA GCACCTGTTA ATGAAAGTTA CATCCCAGAT GAACTCTGTA AGGTCATGGG 360
TATATCGAAA CCACAAATAG TTTTTACGAC AAAGAACATT TTAAATAAGG TATTGGAGGT 420
ACAGAGCAGA ACTAATTTCA TAAAAAGGAT CATCGTACTT GATACTGTAG AAAACATACA 480
CGGTTGTGAA AGTCTTCCCA ATTTTATTTC TCGTTATTCG GATGGAAATA TTGCCAACTT 540
CAAACCTTTA CATTTCGATC CTGTAGAGCA AGTGGCAGCT ATCTTATGTT CGTCAGGCAC 600
TACTGGATTA CCGAAAGGTG TAATGCAAAC TCACCAAAAT ATTTGTGTCC GACTTATACA 660
TGCTTTAGAC CCCAGGGCAG GAACGCAACT TATTCCTGGT GTGACAGTCT TAGTATATCT 720
GCCTTTTTTC CATGCTTTTG GGTTCTCTAT AACCTTGGGA TACTTCATGG TGGGTCTTCG 780
TGTTATCATG TCAAGACGAT TTGATCCAGA AGCATTTCTA AAAGCTATTC AGGATTATGA 840
AGTTCGAAGT GTAATTAACG TTCCATCAGT AATATTGTTC TTATCGAAAA GTCCTTTGGT 900
TGACAAATAC GATTTATCAA GTTTAAGGGA ATTGTGTTGC GGTGCGGCAC CATTAGCAAA 960
AGAAGTTGCT GAGGTTGCAG CAAAACGATT AAACTTGCCA GGAATTCGCT GTGGATTTGG 1020
TTTGACAGAA TCTACTTCAG CTAATATACA CAGTCTTAGG GATGAATTTA AACCAGGATC 1080
ACTTGGAAGA GTTACTCCTT TAATGGCAGC TAAAATAGCA GATAGGGAAA CTGGTAAAGC 1140
ATTGGGACCA AATCAAGTTG GTGAATTATG CATTAAAGGT CCCATGGTAT CGAAAGGTTA 1200
CGTGAACAAT GTAGAAGCTA CCAAAGAAGC TATTGATGAT GATGGTTGGC TTCACTCTGG 1260
AGACTTTGGA TACTATGATG AGGATGAGCA TTTCTATGTG GTGGACCGTT ACAAGGAATT 1320
GATTAAATAT AAGGGCTCTC AGGTAGCACC TGCAGAACTA GAAGAGATTT TATTGAAAAA 1380
TCCATGTATC AGAGATGTTG CTGTGGTTGG TATTCCTGAT CTAGAAGCTG GAGAACTGCC 1440
ATCTGCGTTT GTGGTTAAAC AGCCCGGAAA GGAGATTACA GCTAAAGAAG TGTACGATTA 1500
TCTTGCCGAG AGGGTCTCCC ATACAAAGTA TTTGCGTGGA GGGGTTCGAT TCGTTGATAG 1560
CATACCACGG AATGTTACAG GTAAAATTAC AAGAAAGGAA CTTCTGAAGC AGTTGCTGGA 1620
GAAGGCGGGA GGT
```

FIG. 65

LucPpl 81-0B11

		M	M	K	R	E	K	N	V	I	Y	G	P	E	P	L	H	P	L	18
E	D	L	T	A	G	E	M	L	F	R	A	L	R	K	H	S	H	L	P	38
Q	A	L	V	D	V	Y	G	D	E	S	L	S	Y	K	E	F	F	E	A	58
T	V	L	L	A	Q	S	L	H	N	C	G	Y	K	M	N	D	V	V	S	78
I	C	A	E	N	N	T	R	F	F	I	P	V	I	A	A	W	Y	I	G	98
M	I	V	A	P	V	N	E	S	Y	I	P	D	E	L	C	K	V	M	G	118
I	S	K	P	Q	I	V	F	T	T	K	N	I	L	N	K	V	L	E	V	138
Q	S	R	T	N	F	I	K	R	I	I	V	L	D	T	V	E	N	I	H	158
G	C	E	S	L	P	N	F	I	S	R	Y	S	D	G	N	I	A	N	F	178
K	P	L	H	F	D	P	V	E	Q	V	A	A	I	L	C	S	S	G	T	198
T	G	L	P	K	G	V	M	Q	T	H	Q	N	I	C	V	R	L	I	H	218
A	L	D	P	R	A	G	T	Q	L	I	P	G	V	T	V	L	V	Y	L	238
P	F	F	H	A	F	G	F	S	I	T	L	G	Y	F	M	V	G	L	R	258
V	I	M	S	R	R	F	D	P	E	A	F	L	K	A	I	Q	D	Y	E	278
V	R	S	V	I	N	V	P	S	V	I	L	F	L	S	K	S	P	L	V	298
D	K	Y	D	L	S	S	L	R	E	L	C	C	G	A	A	P	L	A	K	318
E	V	A	E	V	A	A	K	R	L	N	L	P	G	I	R	C	G	F	G	338
L	T	E	S	T	S	A	N	I	H	S	L	R	D	E	F	K	P	G	S	358
L	G	R	V	T	P	L	M	A	A	K	I	A	D	R	E	T	G	K	A	378
L	G	P	N	Q	V	G	E	L	C	I	K	G	P	M	V	S	K	G	Y	398
V	N	N	V	E	A	T	K	E	A	I	D	D	D	G	W	L	H	S	G	418
D	F	G	Y	Y	D	E	D	E	H	F	Y	V	V	D	R	Y	K	E	L	438
I	K	Y	K	G	S	Q	V	A	P	A	E	L	E	E	I	L	L	K	N	458
P	C	I	R	D	V	A	V	V	G	I	P	D	L	E	A	G	E	L	P	478
S	A	F	V	V	K	Q	P	G	K	E	I	T	A	K	E	V	Y	D	Y	498
L	A	E	R	V	S	H	T	K	Y	L	R	G	G	V	R	F	V	D	S	518
I	P	R	N	V	T	G	K	I	T	R	K	E	L	L	K	Q	L	L	E	538
K	A	G	G																	542

FIG. 66

SEQUENCE LISTING

<110> Promega Corporation

<120> Thermostable luciferases and methods of production.

<130> 341.012WO1

<150> US 09/396,154
<151> 1999-09-15

<150> US 09/156,946
<151> 1998-09-18

<150> PCT/US98/19494
<151> 1998-09-18

<150> US 60/059,379
<151> 1997-09-19

<160> 93

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

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catagcattg acaaatgctc atacaaaaga aaatgtttta tatgaagagt ttttaaaatt 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctata attgcatcat tgtatcttgg 300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcataa ttttttgctc caagaatact tttcaaaaag tactgaatgt 420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg 480

aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc tggacgtaaa 540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc 600
tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctcttgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac 720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga 840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc 900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt 960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg 1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaacaatg acgtcagacc 1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg 1140
aaaaattttg gggccaaatg aacctggaga attgtatttt aaaggcgaca tgataatgaa 1200
aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg 1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa 1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt 1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga 1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca 1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt 1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt 1620
tgaaaaacac accaatggg 1639

<210> 2
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> note = "mutant luciferase"

<400> 2

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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt tgttaaaatt 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctata attgcatcat tgtatcttgg 300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt 420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg 480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc tggacgtaaa 540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc 600
tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctcatgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac 720

ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga 840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttttg caaaaagtgc 900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt 960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg 1020
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aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg 1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa 1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt 1380
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gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca 1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt 1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt 1620
tgaaaaacac accaatggg 1639

<210> 3
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> note = "mutant luciferase"

<400> 3
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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt ttttaaaatt 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctata attgcatcat tgtatcttgg 300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt 420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg 480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa 540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc 600
tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgtacgatt 660
ttcttatgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac 720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
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ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc 900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt 960

atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg 1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaacaatg acgtcagacc 1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg 1140
aaaaatttg gggccaaatg aaactggaga attgtatttt aaaggcgaca tgataatgaa 1200
aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg 1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa 1320
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acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga 1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca 1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt 1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt 1620
tgaaaaacac accaatggg 1639

<210> 4

<211> 1639

<212> DNA

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 4

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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt ttttaaaatt 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctata attgcatcat tgtatcttgg 300
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tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt 420
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tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctattgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac 720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga 840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcattttttg caaaaagtgc 900
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gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg 1140
aaaaatttg gggccaaatg aaactggaga attgtatttt aaaggcgaca tgataatgaa 1200

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aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg    1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa    1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt    1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga    1440
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aaatttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt    1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt    1620
tgaaaaacac accaatggg                                                  1639
```

<210> 5
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 5

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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt tgttaaaatt     180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc     240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctata attgcatcat tgtatcttgg     300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg     360
tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt     420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg     480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa     540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc     600
tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgcacgatt     660
ttctcatgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac     720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg     780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga     840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcattttttg caaaaagtgc     900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt     960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg    1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaacaatg acgtcagacc    1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg    1140
aaaaattttg ggccaaatg aaactggaga attgtatttt aaaggcgaca tgataatgaa    1200
aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg    1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa    1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt    1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga    1440
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aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt 1560
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tgaaaaacac accaatggg 1639

<210> 6
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 6
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catagcattg acaaatgctc atacaaaaga aaatgtttta tatgaagagt ttttaaaatt 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctgta attgcatcat tgtatcttgg 300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt 420
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aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc 600
tggtacaact ggtgttccga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctcttgca aaagatccta cttttggtaa cgcaattaat cccacgacag caattttaac 720
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ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc 900
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<210> 7
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<221> unsure
<222> (1067)...(1072)
<223> /note = "unknown nucleotides"

<223> /note = "mutant luciferase"

<400> 7

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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt tttaaaatt      180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc     240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctgta attgcatcat tgtatcttgg     300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg     360
tattgtaaaa ccacgcataa tttttttgctc caagaatact tttcaaaaag tactgaatgt    420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg     480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa     540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc     600
tggtacaact ggtgttccga agggagtcat gctaactcac aagaatattg ttgcacgatt     660
ttctcttgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac     720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg     780
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ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc     900
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atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg    1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaaannnn nngtcagacc    1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg    1140
aaaaattttg ggccaaatg aacctggaga attgtatttt aaaggcgaca tgataatgaa     1200
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acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga    1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca    1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cggggtgggg tgaaattttt    1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt    1620
tgaaaaacac accaatggg                                                  1639
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<210> 8

<211> 1639

<212> DNA

<213> Artificial Sequence

<220>

<221> unsure

<222> (1067)...(1072)

<223> /note = "unknown nucleotides"

<223> note = " Mutant luciferase

<400> 8

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tgggacggct ggagaacaga tgttttacgc attatctcgt tatgcagata tttcaggatg    120
catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt tgttaaaatt    180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc    240
ggtgtgtagc gaaaatggtt tgcaatattt ccttcctgta attgcatcat tgtatcttgg    300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg    360
tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt    420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg    480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa    540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc    600
tggtacaact ggtgttccga agggagtcat gctaactcac aagaatattg ttgcacgatt    660
ttctattgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac    720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg    780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga    840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc    900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt    960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg   1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaaannnn nngccagacc   1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg   1140
aaaaattttg ggccaaatg aacctggaga attgtatttt aaaggcgcca tgataatgaa   1200
gggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg   1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa   1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt   1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga   1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca   1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt   1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt   1620
tgaaaaacac accaatggg                                                 1639
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<210> 9
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<221> unsure
<222> (1067)...(1072)
<223> /note = "unknown nucleotides"

<223> note = " Mutant luciferase

<400> 9

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tgggacggct ggagaacaga tgtttgacgc attatctcgt tatgcagata tttcaggatg    120
catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt tgttaaaatt    180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc    240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctgta attgcatcat tgtatcttgg    300
aataattgca gcacctgtta gtgataaata cgttgaacgt gaattaatac acagtcttgg    360
tattgtaaaa ccacgcataa tttttgctc caagaatact tttcaaaaag tactgaatgt    420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg    480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatagtaatc tggacgtaaa    540
aaaatttaaa ccaaattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc    600
tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgcacgatt    660
ttctcttgca aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac    720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg    780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga    840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc    900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt    960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg   1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaacnnnn nngccagacc   1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg   1140
aaaaatttg gggccaaatg aacctggaga attgtatttt aaaggcgcca tgataatgaa    1200
gggttattat aataatgaag aagctactaa agcaattatt gataaagacg gatggttgcg   1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa   1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt   1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga   1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca   1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt   1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt   1620
tgaaaaacac accaatggg                                                1639
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<210> 10
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<221> unsure
<222> (1067)...(1072)
<223> /note = "unknown nucleotides"

<223> note = " Mutant luciferase

<400> 10

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tgggacggct ggagaacaga tgttttacgc attatctcgt tatgcagata ttccgggctg     120
catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt ttttaaaatt     180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc     240
ggtgtgtagc gaaaatggtt tgcaattttt ccttcctgta attgcatcat tgtatcttgg     300
aataattgtg gcacctgtta acgataaata cattgaacgt gaattaatac acagtcttgg     360
tattgtaaaa ccacgcatag tttttgctc caagaatact tttcaaaaag tactgaatgt      420
aaaatctaaa ttaaaatctg tagaaactat tattatatta gacttaaatg aagacttagg     480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa     540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttgatta tgttttcttc     600
tggtacaact ggtctgccga agggagtcat gctaactcac aagaatattg ttgcacgatt     660
ttctcttgca aaagatccta cttttggtaa cgcaattaat cccacgacag caattttaac     720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg     780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga     840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc     900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt     960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg    1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaaannnn nngccagacc    1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg    1140
aaaaattttg ggccaaatg aacctggaga attgtatttt aaaggcccga tgataatgaa    1200
gggttattat aataatgaag aagctactaa agcaattatt gataatgacg gatggttgcg    1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa    1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt    1380
acaacatccg tatattgttg atgccggcgt tactggtatt ccggatgaag ccgcgggcga    1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca    1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt    1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt    1620
tgaaaaacac accaatggg                                                 1639
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<210> 11
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 11
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catagcattg acaaatgctc atacaaaaga aaatgtttta tatgaagagt ttctgaaact 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatggtc tgcaattttt ccttcctgta attgcatcat tgtatcttgg 300
aataattgtg gcacctgtta acgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcatag ttttttgctc caagaatact tttcaaaaag tactgaatgt 420
aaaatctaaa ttaaaatcta ttgaaactat tattatatta gacttaaatg aagacttagg 480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatagtaatc tggacgtaaa 540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttgatta tgttttcttc 600
tggtacaact ggtctgccga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctcttgca aaagatccta cttttggtaa cgcaattaat cccacgacag caattttaac 720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga 840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc 900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt 960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg 1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaaaggtg acgccaaacc 1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg 1140
aaaaattttg ggccaaatg aacctggaga attgtatttt aaaggcccga tgataatgaa 1200
gggttattat aataatgaag aagctactaa agcaattatt gataatgacg gatggttgcg 1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa 1320
gtcactgatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt 1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga 1440
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agattatgtt gccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt 1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt 1620
tgaaaaacac accaatggg 1639

<210> 12
<211> 1642
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 12

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catagcattg acaaatgctc atacaaaaga aaatgtttta tatgaagagt tttaaaaatt    180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc    240
ggtgtgtagc gaaaatggtt tgcaattttt ccttccttta attgcatcat tgtatcttgg    300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg    360
tattgtaaaa ccacgcataa ttttttgttc caagaatact tttcaaaaag tactgaatgt    420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg    480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa    540
aaaatttaaa ccaaattctt ttaatcgaga cgatcaggtt gcgttggtaa tgttttcttc    600
tggtacaact ggtgtttcga agggagtcat gctaactcac aagaatattg ttgcacgatt    660
ttctcattgc aaagatccta cttttggtaa cgcaattaat ccaacgacag caattttaac    720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg    780
attccgagtt gctctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga    840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcattttttg caaaaagtgc    900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt    960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg   1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccggacactg acgtcagacc   1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg   1140
aaaaattttg ggccaaatg aaactggaga attgtatttt aaaggcgaca tgataatgaa    1200
aagttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg   1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa   1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt   1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga   1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca   1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt   1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt   1620
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<210> 13
<211> 1626
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 13

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ttagtagatg tggttggcga cgaatcgctt tcctataaag agtttttga agcgacagtc      180
ctcctagcgc aaagtctcca caattgtgga tacaagatga atgatgtagt gtcgatctgc     240
gccgagaata atacaagatt ttttattccc gttattgcag cttggtatat tggtatgatt     300
gtagcacctg ttaatgaaag ttacatccca gatgaactct gtaaggtgat gggtatatcg     360
aaaccacaaa tagtttttac gacaaagaac attttaaata aggtattgga ggtacagagc     420
agaactaatt tcataaaaag gatcatcata cttgatactg tagaaaacat acacggttgt     480
gaaagtcttc ccaattttat ttctcgttat tcggatggaa atattgccaa cttcaaacct     540
ttacatttcg atcctgttga gcaagtggca gctatcttat gttcgtcagg cactactgga     600
ttaccgaaag gtgtaatgca aactcaccaa aatatttgtg tccgacttat acatgcttta     660
gaccccaggg caggaacgca acttattcct ggtgtgacag tcttagtata tctgcctttt     720
ttccatgctt ttgggttctc tataaccttg ggatacttca tggtgggtct tcgtgttatc     780
atgttcagac gatttgatca agaagcattt ctaaaagcta ttcaggatta tgaagttcga     840
agtgtaatta acgttccatc agtaatattg ttcttatcga aaagtccttt ggttgacaaa     900
tacgatttat caagtttaag ggaattgtgt tgcggtgcgg caccattagc aaaagaagtt     960
gctgaggttg cagcaaaacg attaaacttg ccaggaattc gctgtggatt tggtttgaca    1020
gaatctactt cagctaatat acacagtctt agggatgaat ttaaatcagg atcacttgga    1080
agagttactc ctttaatggc agctaaaata gcagataggg aaactggtaa agcattggga    1140
ccaaatcaag ttggtgaatt atgcattaaa ggtcccatgg tatcgaaagg ttacgtgaac    1200
aatgtagaag ctaccaaaga agctattgat gatgatggtt ggcttcactc tggagacttt    1260
ggatactatg atgaggatga gcatttctat gtggtggacc gttacaagga attgattaaa    1320
tataagggct ctcaggtagc acctgcagaa ctagaagaga ttttattgaa aaatccatgt    1380
atcagagatg ttgctgtggt tggtattcct gatctagaag ctggagaact gccatctgcg    1440
tttgtggtta aacagcccgg aaaggagatt acagctaaag aagtgtacga ttatcttgcc    1500
gagagggtct cccatacaaa gtatttgcgt ggaggggttc gattcgttga tagcatacca    1560
aggaatgtta caggtaaaat tacaagaaag gaacttctga agcagttgct ggagaaggcg    1620
ggaggt                                                               1626
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<210> 14

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 14

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1               5                   10                  15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
            20                  25                  30
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Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
340 345 350

Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
370 375 380

Glu Pro Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr
385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp
405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495

Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530 535 540

<210> 15
<211> 544
<212> PRT
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 15
Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Leu Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
340 345 350

Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr
385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp
405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
420 425 430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530 535 540

<210> 16
<211> 544
<212> PRT
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 16
Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
20 25 30
Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr
85 90 95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Val Arg Phe Ser Leu Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
340 345 350

Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr
385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Thr Lys Asp Gly Trp
405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530 535 540

<210> 17
<211> 544
<212> PRT
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 17
Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
20 25 30
Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr
85 90 95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Ile Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
340 345 350

Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr
385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp
405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495

Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530 535 540

<210> 18

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 18

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
340 345 350

Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr
385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp
405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495

Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530 535 540

<210> 19

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 19

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
340 345 350

Lys Gly Asp Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
370 375 380

Glu Pro Gly Glu Leu Tyr Phe Lys Gly Ala Met Ile Met Lys Gly Tyr
385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp
405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495

Val Gln Asp Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530 535 540

<210> 20

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<221> unsure

<222> (354)...(355)

<223> /note = "unknown amino acids"

<400> 20

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp

290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys

305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg

325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro

340 345 350

Lys Xaa Xaa Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His

355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Pro Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Lys Asp Gly Trp

405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val

450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro

465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile

485 490 495

Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg

500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys

515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly

530 535 540

<210> 21

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<221> unsure

<222> (354)...(355)

<223> /note = "unknown amino acids"

<400> 21

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Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1               5                   10                  15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
            20                  25                  30
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
        35                  40                  45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
    50                  55                  60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65                  70                  75                  80
Ser Glu Asn Gly Leu Gln Tyr Phe Leu Pro Val Ile Ala Ser Leu Tyr
                85                  90                  95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
            100                 105                 110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
        115                 120                 125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
    130                 135                 140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145                 150                 155                 160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
                165                 170                 175
Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala
            180                 185                 190
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
        195                 200                 205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Ile Ala Lys Asp Pro
    210                 215                 220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225                 230                 235                 240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
                245                 250                 255
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
            260                 265                 270
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
        275                 280                 285
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
    290                 295                 300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
```

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305                 310                 315                 320
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
                325                 330                 335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
            340                 345                 350
Lys Xaa Xaa Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
        355                 360                 365
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
    370                 375                 380
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Ala Met Ile Met Lys Gly Tyr
385                 390                 395                 400
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Lys Asp Gly Trp
                405                 410                 415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
            420                 425                 430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
        435                 440                 445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
    450                 455                 460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465                 470                 475                 480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
                485                 490                 495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
            500                 505                 510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
        515                 520                 525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
    530                 535                 540
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<210> 22
<211> 544
<212> PRT
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<221> unsure
<222> (354)...(355)
<223> /note = "unknown amino acids"

<400> 22

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
20 25 30

Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Val Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
165 170 175

Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285

Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg

325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro

340 345 350

Lys Xaa Xaa Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His

355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Ala Met Ile Met Lys Gly Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Lys Asp Gly Trp

405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val

450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro

465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile

485 490 495

Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg

500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys

515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly

530 535 540

<210> 23

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<221> unsure

<222> (354)...(355)

<223> /note = "unknown amino acids"

<400> 23

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu

1 5 10 15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
20 25 30
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
85 90 95
Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu
100 105 110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser
130 135 140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145 150 155 160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
165 170 175
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190
Leu Ile Met Phe Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met
195 200 205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
210 215 220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225 230 235 240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
245 250 255
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
260 265 270
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
275 280 285
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
290 295 300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
325 330 335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro

340 345 350

Lys Xaa Xaa Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His

355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Pro Gly Glu Leu Tyr Phe Lys Gly Pro Met Ile Met Lys Gly Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp

405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val

450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro

465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile

485 490 495

Val Gln Asp Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg

500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys

515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly

530 535 540

<210> 24

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 24

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu

1 5 10 15

Glu Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr

20 25 30

Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu

35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu

50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys

65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr

85 90 95

Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu

100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Val Phe Cys Ser

115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser

130 135 140

Ile Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr

145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp

165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala

180 185 190

Leu Ile Met Phe Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met

195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro

210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile

225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr

245 250 255

Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe

260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro

275 280 285

Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp

290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys

305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg

325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro

340 345 350

Lys Gly Asp Ala Lys Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His

355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Pro Gly Glu Leu Tyr Phe Lys Gly Pro Met Ile Met Lys Gly Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp

405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val

450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro

465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile

485 490 495

Val Gln Asp Tyr Val Ala Ser Gln Val Ser Thr Ala Lys Trp Leu Arg

500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys

515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly

530 535 540

<210> 25

<211> 545

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 25

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu

1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr

20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu

35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu

50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys

65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Leu Ile Ala Ser Leu Tyr

85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu

100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser

115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr

130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr

145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp

165 170 175

Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala

180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met

195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Cys Lys Asp Pro

210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile

225 230 235 240

Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr

245 250 255

Cys Gly Phe Arg Val Ala Leu Met His Thr Phe Glu Glu Lys Leu Phe

260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro

275 280 285

Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp

290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys

305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg

325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro

340 345 350

Asp Thr Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His

355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp

405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

435 440 445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
450 455 460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465 470 475 480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
485 490 495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
500 505 510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
515 520 525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Lys Ser Lys
530 535 540
Leu
545

<210> 26
<211> 542
<212> PRT
<213> Artificial Sequence

<220>
<223> note = "mutant luciferase"

<400> 26
Met Met Lys Arg Glu Lys Asn Val Ile Tyr Gly Pro Glu Pro Leu His
1 5 10 15
Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg
20 25 30
Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Val Gly Asp Glu
35 40 45
Ser Leu Ser Tyr Lys Glu Phe Phe Glu Ala Thr Val Leu Leu Ala Gln
50 55 60
Ser Leu His Asn Cys Gly Tyr Lys Met Asn Asp Val Val Ser Ile Cys
65 70 75 80
Ala Glu Asn Asn Thr Arg Phe Phe Ile Pro Val Ile Ala Ala Trp Tyr
85 90 95
Ile Gly Met Ile Val Ala Pro Val Asn Glu Ser Tyr Ile Pro Asp Glu
100 105 110
Leu Cys Lys Val Met Gly Ile Ser Lys Pro Gln Ile Val Phe Thr Thr
115 120 125
Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asn Phe

130 135 140

Ile Lys Arg Ile Ile Ile Leu Asp Thr Val Glu Asn Ile His Gly Cys

145 150 155 160

Glu Ser Leu Pro Asn Gly Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala

165 170 175

Asn Phe Lys Pro Leu His Phe Asp Pro Val Glu Gln Val Ala Ala Ile

180 185 190

Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr

195 200 205

His Gln Asn Ile Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Ala

210 215 220

Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe

225 230 235 240

Phe His Ala Phe Gly Phe Ser Ile Thr Leu Gly Tyr Phe Met Val Gly

245 250 255

Leu Arg Val Ile Met Phe Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys

260 265 270

Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ser Val

275 280 285

Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser

290 295 300

Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val

305 310 315 320

Ala Glu Val Ala Ala Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly

325 330 335

Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Arg Asp

340 345 350

Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala

355 360 365

Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val

370 375 380

Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn

385 390 395 400

Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His

405 410 415

Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val

420 425 430

Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro

435 440 445

Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val

450 455 460

Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala

465 470 475 480
Phe Val Val Lys Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr
485 490 495
Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly
500 505 510
Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr
515 520 525
Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ala Gly Gly
530 535 540

<210> 27
<211> 548
<212> PRT
<213> Luciola cruciata

<400> 27
Met Glu Asn Met Glu Asn Asp Glu Asn Ile Val Val Gly Pro Lys Pro
1 5 10 15
Phe Tyr Pro Ile Glu Glu Gly Ser Ala Gly Thr Gln Leu Arg Lys Tyr
20 25 30
Met Glu Arg Tyr Ala Lys Leu Gly Ala Ile Ala Phe Thr Asn Ala Val
35 40 45
Thr Gly Val Asp Tyr Ser Tyr Ala Glu Tyr Leu Glu Lys Ser Cys Cys
50 55 60
Leu Gly Lys Ala Leu Gln Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
65 70 75 80
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Ile Ala
85 90 95
Gly Leu Phe Ile Gly Val Gly Val Ala Pro Thr Asn Glu Ile Tyr Thr
100 105 110
Leu Arg Glu Leu Val His Ser Leu Gly Ile Ser Lys Pro Thr Ile Val
115 120 125
Phe Ser Ser Lys Lys Gly Leu Asp Lys Val Ile Thr Val Gln Lys Thr
130 135 140
Val Thr Thr Ile Lys Thr Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
145 150 155 160
Arg Gly Tyr Gln Cys Leu Asp Thr Phe Ile Lys Arg Asn Thr Pro Pro
165 170 175
Gly Phe Gln Ala Ser Ser Phe Lys Thr Val Glu Val Asp Arg Lys Glu
180 185 190
Gln Val Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys
195 200 205

Gly Val Gln Leu Thr His Glu Asn Thr Val Thr Arg Phe Ser His Ala
210 215 220

Arg Asp Pro Ile Tyr Gly Asn Gln Val Ser Pro Gly Thr Ala Val Leu
225 230 235 240

Thr Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly
245 250 255

Tyr Leu Ile Cys Gly Phe Arg Val Val Met Leu Thr Lys Phe Asp Glu
260 265 270

Glu Thr Phe Leu Lys Thr Leu Gln Asp Tyr Lys Cys Thr Ser Val Ile
275 280 285

Leu Val Pro Thr Leu Phe Ala Ile Leu Asn Lys Ser Glu Leu Leu Asn
290 295 300

Lys Tyr Asp Leu Ser Asn Leu Val Glu Ile Ala Ser Gly Gly Ala Pro
305 310 315 320

Leu Ser Lys Glu Val Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro
325 330 335

Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile
340 345 350

Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val
355 360 365

Pro Leu Phe Lys Ala Lys Val Ile Asp Leu Asp Thr Lys Lys Ser Leu
370 375 380

Gly Pro Asn Arg Arg Gly Glu Val Cys Val Lys Gly Pro Met Leu Met
385 390 395 400

Lys Gly Tyr Val Asn Asn Pro Glu Ala Thr Lys Glu Leu Ile Asp Glu
405 410 415

Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Glu Lys
420 425 430

His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly
435 440 445

Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro
450 455 460

Ser Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Val Ala Gly
465 470 475 480

Glu Leu Pro Gly Ala Val Val Val Leu Glu Ser Gly Lys Asn Met Thr
485 490 495

Glu Lys Glu Val Met Asp Tyr Val Ala Ser Gln Val Ser Asn Ala Lys
500 505 510

Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu
515 520 525

Thr Gly Lys Ile Asp Gly Arg Ala Ile Arg Glu Ile Leu Lys Lys Pro
530 535 540

Val Ala Lys Met
545

<210> 28
<211> 548
<212> PRT
<213> Luciola lateralis

<400> 28
Met Glu Asn Met Glu Asn Asp Glu Asn Ile Val Tyr Gly Pro Glu Pro
1 5 10 15
Phe Tyr Pro Ile Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg Lys Tyr
20 25 30
Met Asp Arg Tyr Ala Lys Leu Gly Ala Ile Ala Phe Thr Asn Ala Leu
35 40 45
Thr Gly Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu Lys Ser Cys Cys
50 55 60
Leu Gly Glu Ala Leu Lys Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
65 70 75 80
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Leu Ala
85 90 95
Gly Leu Phe Ile Gly Val Gly Val Ala Pro Thr Asn Glu Ile Tyr Thr
100 105 110
Leu Arg Glu Leu Val His Ser Leu Gly Ile Ser Lys Pro Thr Ile Val
115 120 125
Phe Ser Ser Lys Lys Gly Leu Asp Lys Val Ile Thr Val Gln Lys Thr
130 135 140
Val Ala Thr Ile Lys Thr Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
145 150 155 160
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile Lys Lys Asn Thr Pro Gln
165 170 175
Gly Phe Lys Gly Ser Ser Phe Lys Thr Val Glu Val Asn Arg Lys Glu
180 185 190
Gln Val Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys
195 200 205
Gly Val Gln Leu Thr His Glu Asn Ala Val Thr Arg Phe Ser His Ala
210 215 220
Arg Asp Pro Ile Tyr Gly Asn Gln Val Ser Pro Gly Thr Ala Ile Leu
225 230 235 240
Thr Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly
245 250 255
Tyr Leu Thr Cys Gly Phe Arg Ile Val Met Leu Thr Lys Phe Asp Glu

260 265 270

Glu Thr Phe Leu Lys Thr Leu Gln Asp Tyr Lys Cys Ser Ser Val Ile

275 280 285

Leu Val Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser Glu Leu Leu Asp

290 295 300

Lys Tyr Asp Leu Ser Asn Leu Val Glu Ile Ala Ser Gly Gly Ala Pro

305 310 315 320

Leu Ser Lys Glu Ile Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro

325 330 335

Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile

340 345 350

Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val

355 360 365

Pro Leu Phe Lys Ala Lys Val Ile Asp Leu Asp Thr Lys Lys Thr Leu

370 375 380

Gly Pro Asn Arg Arg Gly Glu Val Cys Val Lys Gly Pro Met Leu Met

385 390 395 400

Lys Gly Tyr Val Asp Asn Pro Glu Ala Thr Arg Glu Ile Ile Asp Glu

405 410 415

Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Glu Lys

420 425 430

His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly

435 440 445

Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro

450 455 460

Asn Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Ile Ala Gly

465 470 475 480

Glu Leu Pro Gly Ala Val Val Val Leu Glu Lys Gly Lys Ser Met Thr

485 490 495

Glu Lys Glu Val Met Asp Tyr Val Ala Ser Gln Val Ser Asn Ala Lys

500 505 510

Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu

515 520 525

Thr Gly Lys Ile Asp Gly Lys Ala Ile Arg Glu Ile Leu Lys Lys Pro

530 535 540

Val Ala Lys Met

545

<210> 29

<211> 548

<212> PRT

<213> Luciola mingrelica

<400> 29

Met Glu Met Glu Lys Glu Glu Asn Val Val Tyr Gly Pro Leu Pro Phe
1 5 10 15

Tyr Pro Ile Glu Glu Gly Ser Ala Gly Ile Gln Leu His Lys Tyr Met
20 25 30

His Gln Tyr Ala Lys Leu Gly Ala Ile Ala Phe Ser Asn Ala Leu Thr
35 40 45

Gly Val Asp Ile Ser Tyr Gln Glu Tyr Phe Asp Ile Thr Cys Arg Leu
50 55 60

Ala Glu Ala Met Lys Asn Phe Gly Met Lys Pro Glu Glu His Ile Ala
65 70 75 80

Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Leu Ala Gly
85 90 95

Leu Tyr Ile Gly Val Ala Val Ala Pro Thr Asn Glu Ile Tyr Thr Leu
100 105 110

Arg Glu Leu Asn His Ser Leu Gly Ile Ala Gln Pro Thr Ile Val Phe
115 120 125

Ser Ser Arg Lys Gly Leu Pro Lys Val Leu Glu Val Gln Lys Thr Val
130 135 140

Thr Cys Ile Lys Lys Ile Val Ile Leu Asp Ser Lys Val Asn Phe Gly
145 150 155 160

Gly His Asp Cys Met Glu Thr Phe Ile Lys Lys His Val Glu Leu Gly
165 170 175

Phe Gln Pro Ser Ser Phe Val Pro Ile Asp Val Lys Asn Arg Lys Gln
180 185 190

His Val Ala Leu Leu Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys
195 200 205

Gly Val Arg Ile Thr His Glu Gly Ala Val Thr Arg Phe Ser His Ala
210 215 220

Lys Asp Pro Ile Tyr Gly Asn Gln Val Ser Pro Gly Thr Ala Ile Leu
225 230 235 240

Thr Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly
245 250 255

Tyr Phe Ala Cys Gly Tyr Arg Val Val Met Leu Thr Lys Phe Asp Glu
260 265 270

Glu Leu Phe Leu Arg Thr Leu Gln Asp Tyr Lys Cys Thr Ser Val Ile
275 280 285

Leu Val Pro Thr Leu Phe Ala Ile Leu Asn Lys Ser Glu Leu Ile Asp
290 295 300

Lys Phe Asp Leu Ser Asn Leu Thr Glu Ile Ala Ser Gly Gly Ala Pro
305 310 315 320

Leu Ala Lys Glu Val Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro

325 330 335

Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Phe Ile

340 345 350

Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val

355 360 365

Pro Leu Phe Lys Val Lys Val Ile Asp Leu Asp Thr Lys Lys Thr Leu

370 375 380

Gly Val Asn Arg Arg Gly Glu Ile Cys Val Lys Gly Pro Ser Leu Met

385 390 395 400

Leu Gly Tyr Ser Asn Asn Pro Glu Ala Thr Arg Glu Thr Ile Asp Glu

405 410 415

Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Asp Glu

420 425 430

His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly

435 440 445

Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro

450 455 460

Asn Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Asp Ala Gly

465 470 475 480

Glu Leu Pro Gly Ala Val Val Val Met Glu Lys Gly Lys Thr Met Thr

485 490 495

Glu Lys Glu Ile Val Asp Tyr Val Asn Ser Gln Val Val Asn His Lys

500 505 510

Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu

515 520 525

Thr Gly Lys Ile Asp Ala Lys Val Ile Arg Glu Ile Leu Lys Lys Pro

530 535 540

Gln Ala Lys Met

545

<210> 30

<211> 548

<212> PRT

<213> Pyrocoelia miyako

<400> 30

Met Glu Asp Asp Ser Lys His Ile Met His Gly His Arg His Ser Ile

1 5 10 15

Leu Trp Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys

20 25 30

Arg Tyr Ala Gln Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ala

35 40 45

Glu Val Asn Ile Thr Tyr Ser Glu Tyr Phe Glu Met Ser Cys Arg Leu
50 55 60
Ala Glu Thr Met Lys Arg Tyr Gly Leu Gly Leu Gln His His Ile Ala
65 70 75 80
Val Cys Ser Glu Thr Ser Leu Gln Phe Phe Met Pro Val Cys Gly Ala
85 90 95
Leu Phe Ile Gly Val Gly Val Ala Pro Thr Asn Asp Ile Tyr Asn Glu
100 105 110
Arg Glu Leu Tyr Asn Ser Leu Phe Ile Ser Gln Pro Thr Ile Val Phe
115 120 125
Cys Ser Lys Arg Ala Leu Gln Lys Ile Leu Gly Val Gln Lys Lys Leu
130 135 140
Pro Val Ile Gln Lys Ile Val Ile Leu Asp Ser Arg Glu Asp Tyr Met
145 150 155 160
Gly Lys Gln Ser Met Tyr Ser Phe Ile Glu Ser His Leu Pro Ala Gly
165 170 175
Phe Asn Glu Tyr Asp Tyr Ile Pro Asp Ser Phe Asp Arg Glu Thr Ala
180 185 190
Thr Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly
195 200 205
Val Asp Leu Thr His Met Asn Val Cys Val Arg Phe Ser His Cys Arg
210 215 220
Asp Pro Val Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Thr
225 230 235 240
Val Ile Pro Phe His His Val Phe Gln Met Phe Thr Thr Leu Gly Tyr
245 250 255
Leu Thr Cys Gly Phe Arg Ile Val Leu Met Tyr Arg Phe Glu Glu Glu
260 265 270
Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu
275 280 285
Val Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Val Asp Lys
290 295 300
Tyr Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu
305 310 315 320
Ala Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe Lys Leu Pro Gly
325 330 335
Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile Ile
340 345 350
Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Cys Gly Lys Val Val Pro
355 360 365
Phe Phe Thr Ala Lys Ile Val Asp Leu Asp Thr Gly Lys Thr Leu Gly
370 375 380

Val Asn Gln Arg Gly Glu Leu Cys Val Lys Gly Pro Met Ile Met Lys
385 390 395 400

Gly Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp
405 410 415

Gly Trp Leu His Ser Gly Asp Ile Ala Tyr Tyr Asp Lys Asp Gly His
420 425 430

Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr
435 440 445

Gln Val Pro Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Phe
450 455 460

Ile Phe Asp Ala Gly Val Ala Gly Ile Pro Asp Pro Asp Ala Gly Glu
465 470 475 480

Leu Pro Ala Ala Val Val Val Leu Glu Glu Gly Lys Met Met Thr Glu
485 490 495

Gln Glu Val Met Asp Tyr Val Ala Gly Gln Val Thr Ala Ser Lys Arg
500 505 510

Leu Arg Gly Gly Val Lys Phe Val Asp Glu Val Pro Lys Gly Leu Thr
515 520 525

Gly Lys Ile Asp Ser Arg Lys Ile Arg Glu Ile Leu Thr Met Gly Gln
530 535 540

Lys Ser Lys Leu
545

<210> 31
<211> 550
<212> PRT
<213> Photinus pyralis

<400> 31

Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1 5 10 15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20 25 30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
35 40 45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50 55 60

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg

100 105 110
Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125
Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140
Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160
Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175
Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190
Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205
Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220
Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240
Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255
Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
260 265 270
Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285
Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300
Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320
Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335
Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350
Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365
Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380
Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400
Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415
Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430
Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln

435 440 445
Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460
Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465 470 475 480
Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495
Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510
Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525
Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540
Gly Gly Lys Ser Lys Leu
545 550

<210> 32
<211> 547
<212> PRT
<213> Lampyris noctiluca

<400> 32
Met Glu Asp Ala Lys Asn Ile Met His Gly Pro Ala Pro Phe Tyr Pro
1 5 10 15
Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20 25 30
Tyr Ala Gln Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ala Glu
35 40 45
Val Asn Ile Thr Tyr Ser Glu Tyr Phe Glu Met Ala Cys Arg Leu Ala
50 55 60
Glu Thr Met Lys Arg Tyr Gly Leu Gly Leu Gln His His Ile Ala Val
65 70 75 80
Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Cys Gly Ala Leu
85 90 95
Phe Ile Gly Val Gly Val Ala Ser Thr Asn Asp Ile Tyr Asn Glu Arg
100 105 110
Glu Leu Tyr Asn Ser Leu Ser Ile Ser Gln Pro Thr Ile Val Ser Cys
115 120 125
Ser Lys Arg Ala Leu Gln Lys Ile Leu Gly Val Gln Lys Lys Leu Pro
130 135 140
Ile Ile Gln Lys Ile Val Ile Leu Asp Ser Arg Glu Asp Tyr Met Gly
145 150 155 160

Lys Gln Ser Met Tyr Ser Phe Ile Glu Ser His Leu Pro Ala Gly Phe
165 170 175

Asn Glu Tyr Asp Tyr Ile Pro Asp Ser Phe Asp Arg Glu Thr Ala Thr
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Glu Leu Thr His Gln Asn Val Cys Val Arg Phe Ser His Cys Arg Asp
210 215 220

Pro Val Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Thr Val
225 230 235 240

Ile Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Thr Cys Gly Phe Arg Ile Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Val Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ala
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe Lys Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile Ile Thr
340 345 350

Pro Glu Gly Asp Asp Lys Pro Gly Ala Cys Gly Lys Val Val Pro Phe
355 360 365

Phe Ser Ala Lys Ile Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Lys Gly Pro Met Ile Met Lys Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Ser Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Tyr Asp Lys Asp Gly His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Pro Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Phe Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Ile Pro Asp Pro Asp Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Val Val Val Leu Glu Glu Gly Lys Thr Met Thr Glu Gln
485 490 495

Glu Val Met Asp Tyr Val Ala Gly Gln Val Thr Ala Ser Lys Arg Leu
500 505 510

Arg Gly Gly Val Lys Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Ile Asp Gly Arg Lys Ile Arg Glu Ile Leu Met Met Gly Lys Lys
530 535 540

Ser Lys Leu
545

<210> 33

<211> 552

<212> PRT

<213> Photuris pennsylvanica

<400> 33

Met Ser Ile Glu Asn Asn Ile Leu Ile Gly Pro Pro Pro Tyr Tyr Pro
1 5 10 15

Leu Glu Glu Gly Thr Ala Gly Glu Gln Leu His Arg Ala Ile Ser Arg
20 25 30

Tyr Ala Ala Val Pro Gly Thr Leu Ala Tyr Thr Asp Val His Thr Glu
35 40 45

Leu Glu Val Thr Tyr Lys Glu Phe Leu Asp Val Thr Cys Arg Leu Ala
50 55 60

Glu Ala Met Lys Asn Tyr Gly Leu Gly Leu Gln His Thr Ile Ser Val
65 70 75 80

Cys Ser Glu Asn Cys Val Gln Phe Phe Met Pro Ile Cys Ala Ala Leu
85 90 95

Tyr Val Gly Val Ala Thr Ala Pro Thr Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Tyr Asn Ser Leu Ser Ile Ser Gln Pro Thr Val Val Phe Thr
115 120 125

Ser Arg Asn Ser Leu Gln Lys Ile Leu Gly Val Gln Ser Arg Leu Pro
130 135 140

Ile Ile Lys Lys Ile Ile Ile Leu Asp Gly Lys Lys Asp Tyr Leu Gly
145 150 155 160

Tyr Gln Ser Met Gln Ser Phe Met Lys Glu His Val Pro Ala Asn Phe
165 170 175

Asn Val Ser Ala Phe Lys Pro Leu Ser Phe Asp Leu Asp Arg Val Ala
180 185 190

Cys Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val Pro
195 200 205

Ile Ser His Arg Asn Thr Ile Tyr Arg Phe Ser His Cys Arg Asp Pro

210 215 220
Val Phe Gly Asn Gln Ile Ile Pro Asp Thr Thr Ile Leu Cys Ala Val
225 230 235 240
Pro Phe His His Ala Phe Gly Thr Phe Thr Asn Leu Gly Tyr Leu Ile
245 250 255
Cys Gly Phe His Val Val Leu Met Tyr Arg Phe Asn Glu His Leu Phe
260 265 270
Leu Gln Thr Leu Gln Asp Tyr Lys Cys Gln Ser Ala Leu Leu Val Pro
275 280 285
Thr Val Leu Ala Phe Leu Ala Lys Asn Pro Leu Val Asp Lys Tyr Asp
290 295 300
Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305 310 315 320
Glu Ile Ser Glu Ile Ala Ala Lys Arg Phe Lys Leu Pro Gly Ile Arg
325 330 335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Cys Ala Ile Val Ile Thr Ala
340 345 350
Glu Gly Glu Phe Lys Leu Gly Ala Val Gly Lys Val Val Pro Phe Tyr
355 360 365
Ser Leu Lys Val Leu Asp Leu Asn Thr Gly Lys Lys Leu Gly Pro Asn
370 375 380
Glu Arg Gly Glu Ile Cys Phe Lys Gly Pro Met Ile Met Lys Gly Tyr
385 390 395 400
Ile Asn Asn Pro Glu Ala Thr Arg Glu Leu Ile Asp Glu Glu Gly Trp
405 410 415
Ile His Ser Gly Asp Ile Gly Tyr Phe Asp Glu Asp Gly His Val Tyr
420 425 430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
435 440 445
Pro Pro Ala Glu Leu Glu Ala Leu Leu Leu Gln His Pro Phe Ile Glu
450 455 460
Asp Ala Gly Val Ala Gly Val Pro Asp Glu Val Ala Gly Asp Leu Pro
465 470 475 480
Gly Ala Val Val Val Leu Lys Glu Gly Lys Ser Ile Thr Glu Lys Glu
485 490 495
Ile Gln Asp Tyr Val Ala Gly Gln Val Thr Ser Ser Lys Lys Leu Arg
500 505 510
Gly Gly Val Glu Phe Val Lys Glu Val Pro Lys Gly Phe Thr Gly Lys
515 520 525
Ile Asp Thr Arg Lys Ile Lys Glu Ile Leu Ile Lys Ala Gln Lys Gly
530 535 540
Lys Ser Lys Ser Lys Ala Lys Leu

545 550

<210> 34

<211> 546

<212> PRT

<213> Phengodes sp.

<400> 34

Met Ile Lys Met Glu Glu Glu His Val Met Pro Gly Ala Met Pro Arg
1 5 10 15

Asp Leu Leu Phe Glu Gly Thr Ala Gly Gln Gln Leu His Arg Ala Leu
20 25 30

Tyr Lys His Ser Tyr Phe Pro Glu Ala Ile Val Asp Ser His Thr His
35 40 45

Glu Ile Ile Ser Tyr Ala Lys Ile Leu Asp Met Ser Cys Arg Leu Ala
50 55 60

Val Ser Phe Gln Lys Tyr Gly Leu Thr Gln Asn Asn Ile Ile Gly Ile
65 70 75 80

Cys Ser Glu Asn Asn Leu Asn Phe Phe Asn Pro Val Ile Ala Ala Phe
85 90 95

Tyr Leu Gly Ile Thr Val Ala Thr Val Asn Asp Thr Tyr Thr Asp Arg
100 105 110

Glu Leu Ser Glu Thr Leu Asn Ile Thr Lys Pro Gln Met Leu Phe Cys
115 120 125

Ser Lys Gln Ser Leu Pro Ile Val Met Lys Thr Met Lys Ile Met Pro
130 135 140

Tyr Val Gln Lys Leu Leu Ile Ile Asp Ser Met Gln Asp Ile Gly Gly
145 150 155 160

Ile Glu Cys Val His Ser Phe Val Ser Arg Tyr Thr Asp Glu His Phe
165 170 175

Asp Pro Leu Lys Phe Val Pro Leu Asp Phe Asp Pro Arg Glu Gln Val
180 185 190

Ala Leu Ile Met Thr Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val
195 200 205

Met Leu Thr His Arg Asn Ile Cys Val Arg Phe Val His Ser Arg Asp
210 215 220

Pro Leu Phe Gly Thr Arg Phe Ile Pro Glu Thr Ser Ile Leu Ser Leu
225 230 235 240

Val Pro Phe His His Ala Phe Gly Met Phe Thr Thr Leu Ser Tyr Phe
245 250 255

Ile Val Gly Leu Lys Ile Val Met Met Lys Arg Phe Asp Gly Glu Leu
260 265 270

Phe Leu Lys Thr Ile Gln Asn Tyr Lys Ile Pro Thr Ile Val Ile Ala
275 280 285

Pro Pro Val Met Val Phe Leu Ala Lys Ser His Leu Val Asp Lys Tyr
290 295 300

Asp Leu Ser Ser Ile Lys Glu Ile Ala Thr Gly Gly Ala Pro Leu Gly
305 310 315 320

Pro Ala Leu Ala Asn Ala Val Ala Lys Arg Leu Lys Leu Gly Gly Ile
325 330 335

Ile Gln Gly Tyr Gly Leu Thr Glu Thr Cys Cys Ala Val Leu Ile Thr
340 345 350

Pro His Asn Lys Ile Lys Thr Gly Ser Thr Gly Gln Val Leu Pro Tyr
355 360 365

Val Thr Ala Lys Ile Val Asp Thr Lys Thr Gly Lys Asn Leu Gly Pro
370 375 380

Asn Gln Thr Gly Glu Leu Cys Phe Lys Ser Asp Ile Ile Met Lys Gly
385 390 395 400

Tyr Tyr Gln Asn Glu Glu Glu Thr Arg Leu Val Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Gly Tyr Tyr Asp Thr Asp Gly Asn Phe
420 425 430

His Ile Val Asp Arg Leu Lys Glu Leu Ile Lys Tyr Lys Ala Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ala Leu Leu Leu Gln His Pro Tyr Ile
450 455 460

Ala Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Glu Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Cys Val Val Leu Glu Pro Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Val Met Asp Tyr Ile Ala Glu Arg Val Thr Pro Thr Lys Arg Leu
500 505 510

Arg Gly Gly Val Leu Phe Val Asn Asn Ile Pro Lys Gly Ala Thr Gly
515 520 525

Lys Leu Val Arg Thr Glu Leu Arg Arg Leu Leu Thr Gln Arg Ala Ala
530 535 540

Lys Leu
545

<210> 35
<211> 543
<212> PRT
<213> Pyrophorus plagiophthalamus

<400> 35

Met Met Lys Arg Glu Lys Asn Val Val Tyr Gly Pro Glu Pro Leu His
1 5 10 15

Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg
20 25 30

Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Tyr Gly Glu Glu
35 40 45

Trp Ile Ser Tyr Lys Glu Phe Phe Glu Thr Thr Cys Leu Leu Ala Gln
50 55 60

Ser Leu His Asn Cys Gly Tyr Lys Met Ser Asp Val Val Ser Ile Cys
65 70 75 80

Ala Glu Asn Asn Lys Arg Phe Phe Val Pro Ile Ile Ala Ala Trp Tyr
85 90 95

Ile Gly Met Ile Val Ala Pro Val Asn Glu Gly Tyr Ile Pro Asp Glu
100 105 110

Leu Cys Lys Val Met Gly Ile Ser Arg Pro Gln Leu Val Phe Cys Thr
115 120 125

Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asp Phe
130 135 140

Ile Lys Arg Ile Ile Ile Leu Asp Ala Val Glu Asn Ile His Gly Cys
145 150 155 160

Glu Ser Leu Pro Asn Phe Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala
165 170 175

Asn Phe Lys Pro Leu His Tyr Asp Pro Val Glu Gln Val Ala Ala Ile
180 185 190

Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr
195 200 205

His Arg Asn Val Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Val
210 215 220

Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe
225 230 235 240

Phe His Ala Phe Gly Phe Ser Ile Asn Leu Gly Tyr Phe Met Val Gly
245 250 255

Leu Arg Val Ile Met Leu Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys
260 265 270

Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ala Ile
275 280 285

Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser
290 295 300

Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val
305 310 315 320

Ala Glu Ile Ala Val Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly

325 330 335

Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Arg Asp

340 345 350

Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala

355 360 365

Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val

370 375 380

Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn

385 390 395 400

Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His

405 410 415

Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val

420 425 430

Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro

435 440 445

Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val

450 455 460

Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala

465 470 475 480

Phe Val Val Ile Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr

485 490 495

Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly

500 505 510

Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr

515 520 525

Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ser Ser Lys Leu

530 535 540

<210> 36

<211> 543

<212> PRT

<213> Pyrophorus plagiophthalamus

<400> 36

Met Met Lys Arg Glu Lys Asn Val Ile Tyr Gly Pro Glu Pro Leu His

1 5 10 15

Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg

20 25 30

Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Phe Gly Asp Glu

35 40 45

Ser Leu Ser Tyr Lys Glu Phe Phe Glu Ala Thr Cys Leu Leu Ala Gln

50 55 60

Ser Leu His Asn Cys Gly Tyr Lys Met Asn Asp Val Val Ser Ile Cys
65 70 75 80

Ala Glu Asn Asn Lys Arg Phe Phe Ile Pro Ile Ile Ala Ala Trp Tyr
85 90 95

Ile Gly Met Ile Val Ala Pro Val Asn Glu Ser Tyr Ile Pro Asp Glu
100 105 110

Leu Cys Lys Val Met Gly Ile Ser Lys Pro Gln Ile Val Phe Cys Thr
115 120 125

Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asn Phe
130 135 140

Ile Lys Arg Ile Ile Ile Leu Asp Thr Val Glu Asn Ile His Gly Cys
145 150 155 160

Glu Ser Leu Pro Asn Phe Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala
165 170 175

Asn Phe Lys Pro Leu His Tyr Asp Pro Val Glu Gln Val Ala Ala Ile
180 185 190

Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr
195 200 205

His Gln Asn Ile Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Ala
210 215 220

Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe
225 230 235 240

Phe His Ala Phe Gly Phe Ser Ile Asn Leu Gly Tyr Phe Met Val Gly
245 250 255

Leu Arg Val Ile Met Leu Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys
260 265 270

Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ala Ile
275 280 285

Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser
290 295 300

Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val
305 310 315 320

Ala Glu Val Ala Val Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly
325 330 335

Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Gly Asp
340 345 350

Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala
355 360 365

Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val
370 375 380

Gly Glu Leu Cys Val Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn
385 390 395 400

Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His
405 410 415

Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val
420 425 430

Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro
435 440 445

Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val
450 455 460

Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala
465 470 475 480

Phe Val Val Lys Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr
485 490 495

Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly
500 505 510

Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr
515 520 525

Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ser Ser Lys Leu
530 535 540

<210> 37

<211> 545

<212> PRT

<213> Photuris pennsylvanica

<400> 37

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Leu Ile Ala Ser Leu Tyr
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr

130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr

145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp

165 170 175

Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala

180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met

195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Cys Lys Asp Pro

210 215 220

Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile

225 230 235 240

Pro Phe His His Gly Phe Gly Met Thr Thr Thr Leu Gly Tyr Phe Thr

245 250 255

Cys Gly Phe Arg Val Ala Leu Met His Thr Phe Glu Glu Lys Leu Phe

260 265 270

Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro

275 280 285

Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp

290 295 300

Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys

305 310 315 320

Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg

325 330 335

Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro

340 345 350

Asp Thr Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His

355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Ser Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp

405 410 415

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

420 425 430

Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

435 440 445

Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val

450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro

465 470 475 480

Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile

485 490 495

Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg

500 505 510

Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys

515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Lys Ser Lys

530 535 540

Leu

545

<210> 38

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> /note = "a primer"

<400> 38

gtactgagac gacgccagcc caagcttagg cctgagtg 38

<210> 39

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> /note = "a primer"

<400> 39

ggcatgagcg tgaactgact gaactagcgg ccgccgag 38

<210> 40

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> /note = "a primer"

<400> 40
gtactgagac gacgccag 18

<210> 41
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "a primer"

<400> 41
ggcatgagcg tgaactgac 19

<210> 42
<211> 1639
<212> DNA
<213> Artificial Sequence

<220>
<223> /note = "mutant luciferase"

<400> 42
agatccaatg gcagataaga atattttata tgggcccgaa ccattttatc ccttggaaga 60
tgggacggct ggagaacaga tgtttgacgc attatctcgt tatgcagata ttccgggctg 120
catagcattg acaaatgctc atacaaaaga aaatgtttta tatgaagagt ttctgaaact 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatagtc tgcaattttt ccttcctgta attgcatcat tgtatcttgg 300
aataattgtg gcacctgtta acgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcatag tttttttgctc caagaatact tttcaaaaag tactgaatgt 420
aaaatctaaa ttaaaatcta ttgaaactat tattatatta gacttaaatg atgacttagg 480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatagtaatc tggacgtaaa 540
aaaatttaaa ccatattctt ttaatcgaga cgatcaggtt gcgttgatta tgttttcttc 600
tggtacaact ggtctgccga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctattgca aaagatccta cttttggtaa cgcaattaat cccacgtcag caattttaac 720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga 840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc 900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt 960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg 1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaaaggtg acgccaaacc 1080
gggatcaact ggtaaaatag taccatttca cgctgttaaa gttgtcgatc ctacaacagg 1140

aaaaattttg gggccaaatg aacctggaga attgtatttt aaaggcccga tgataatgaa 1200
gggttattat aataatgaag aagctactaa agcaattatt gataatgacg gatggttgcg 1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa 1320
gtcactgatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt 1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga 1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca 1500
agattatgtt gccagtcaag tttcaacagc caaatggcta cgtggtgggg tgatattttt 1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt 1620
agaaaaacac accaatggg 1639

<210> 43

<211> 1639

<212> DNA

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 43

ggatccaatg gcagataaga atattttata tgggcccgaa ccattttatc ccttggaaga 60
tgggacggct ggagaacaga tgtttgacgc attatctcgt tatgcagcta ttccgggctg 120
catagcattg acaaatgctc atacaaaaga aaatgtttta tatgaagagt ttctgaaact 180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc 240
ggtgtgtagc gaaaatagtc tgcaattttt ccttcctgta attgcatcat tgtatcttgg 300
aataattgtg gcacctgtta acgataaata cattgaacgt gaattaatac acagtcttgg 360
tattgtaaaa ccacgcatag ttttttgctc caagaatact tttcaaaaag tactgaatgt 420
aaaatctaaa ttaaaatcta ttgaaactat tattatatta gacttaaatg aagacttagg 480
aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatagtaatc tggacgtaaa 540
aaaatttaaa ccctattctt ttaatcgaga cgatcaggtt gcgtcgatta tgttttcttc 600
tggtacaact ggtctgccga agggagtcat gctaactcac aagaatattg ttgcacgatt 660
ttctattgca aaagatccta cttttggtaa cgcaattaat cccacgtcag caattttaac 720
ggtaatacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg 780
attccgagtt gttctaatgc acacgtttga agaaaaacta tttctacaat cattacaaga 840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcatttcttg caaaaagtgc 900
attagttgaa aagtacgatt tatcgcactt aaaagaaatt gcatctggtg gcgcaccttt 960
atcaaaagaa attggggaga tggtgaaaaa acggtttaaa ttaaactttg tcaggcaagg 1020
gtatggatta acagaaacca cttcggctgt tttaattaca ccgaaaggtg acgccaaacc 1080
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115 120 125

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Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
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Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
180 185 190

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Thr Phe Gly Asn Ala Ile Asn Pro Thr Ser Ala Ile Leu Thr Val Ile
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Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
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Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
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Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
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Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
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65                  70                  75                  80
Ser Glu Asn Ser Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
                85                  90                  95
Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu
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    130                 135                 140
Ile Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145                 150                 155                 160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
                165                 170                 175
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
            180                 185                 190
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Thr Phe Gly Asn Ala Ile Asn Pro Thr Ser Ala Ile Leu Thr Val Ile
225                 230                 235                 240
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340 345 350

Lys Gly Asp Ala Lys Pro Gly Ser Thr Gly Lys Ile Val Pro Leu His

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Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn

370 375 380

Glu Pro Gly Glu Leu Tyr Phe Lys Gly Pro Met Ile Met Lys Gly Tyr

385 390 395 400

Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp

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Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr

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Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val

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Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val

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Val Gln Asp Tyr Val Ala Ser Gln Val Ser Thr Ala Lys Trp Leu Arg

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gacagtcctc ctagcgcaaa gtctccacaa ttgtggatac aagatgaatg atgtagtgtc     240
gatctgcgcc gagaataata caagattttt tattcccgtt attgcagctt ggtatattgg     300
tatgattgta gcacctgtta atgaaagtta catcccagat gaactctgta aggtcatggg     360
tatatcgaaa ccacaaatag tttttacgac aaagaacatt ttaaataagg tattggaggt     420
acagagcaga actaatttca taaaaaggat catcgtactt gatactgtag aaaacataca     480
cggttgtgaa agtcttccca attttatttc tcgttattcg gatggaaata ttgccaactt     540
caaaccttta catttcgatc ctgtagagca agtggcagct atcttatgtt cgtcaggcac     600
tactggatta ccgaaaggtg taatgcaaac tcaccaaaat atttgtgtcc gacttataca     660
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tgacaaatac gatttatcaa gtttaaggga attgtgttgc ggtgcggcac cattagcaaa     960
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Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Val Gly Asp Glu

            35   40   45

Ser Leu Ser Tyr Lys Glu Phe Phe Glu Ala Thr Val Leu Leu Ala Gln

        50   55   60

Ser Leu His Asn Cys Gly Tyr Lys Met Asn Asp Val Val Ser Ile Cys

65   70   75   80

Ala Glu Asn Asn Thr Arg Phe Phe Ile Pro Val Ile Ala Ala Trp Tyr

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Ile Gly Met Ile Val Ala Pro Val Asn Glu Ser Tyr Ile Pro Asp Glu

            100   105   110

Leu Cys Lys Val Met Gly Ile Ser Lys Pro Gln Ile Val Phe Thr Thr

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Asn Phe Lys Pro Leu His Phe Asp Pro Val Glu Gln Val Ala Ala Ile

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Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ser Val

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Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser

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Ala Glu Val Ala Ala Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly

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370 375 380

Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn

385 390 395 400

Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His

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Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val

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Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro

435 440 445

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30925

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N15/10 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 14336 A (HALL MARY P ;PROMEGA CORP (US); WOOD KEITH V (US)) 25 March 1999 (1999-03-25) Fig.46,47 page 34, line 1 -page 42, line 6; table 6 page 47, line 3 -page 61, line 2 ---	1-6,8, 12,14, 16,17, 19,21, 23,24, 28-37, 47-58, 65,66, 68,73,75

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 2000	14.08.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 524 448 A (KIKKOMAN CORP) 27 January 1993 (1993-01-27) the whole document ---	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
X	WO 95 25798 A (SECR DEFENCE BRIT ;LOWE CHRISTOPHER ROBIN (GB); WHITE PETER JOHN () 28 September 1995 (1995-09-28) the whole document ---	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
X	WHITE ET AL: "Improved thermostability of the north american firefly luciferase: saturation mutagenesis at position 354" BIOCHEMICAL JOURNAL,GB,PORTLAND PRESS, LONDON, vol. 319, no. 319, 1996, pages 343-350-350, XP002097112 ISSN: 0264-6021 the whole document ---	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
X	WO 98 46729 A (MURRAY JAMES AUGUSTUS HENRY ;SECR DEFENCE (GB); LOWE CHRISTOPHER R) 22 October 1998 (1998-10-22) the whole document ---	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
A	K.V. WOOD ET AL.: "Bioluminescent click beetles revisited" J. BIOLUMNIESCENCE AND CHEMILUMINESCENCE, vol. 4, 1989, pages 31-39, XP000906944 JOHN WILEY & SONS, LTD, NEW YORK, US cited in the application the whole document ---	
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	K.V. WOOD ET AL.: "Complementary DNA coding click beetle luciferases can elict bioluminescence of different colors" SCIENCE, vol. 244, 12 May 1989 (1989-05-12), pages 700-702, XP002137278 AAAS,WASHINGTON,DC,US cited in the application the whole document ---	
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A	L. YE ET AL.: "Cloning and sequencing of a cDNA for firefly luciferase from Photuris pennsylvaniva" BIOCHIMICA BIOPHYSICA ACTA, vol. 1339, 1997, pages 39-52, XP000909154 ELSEVIER SCIENCE, AMSTERDAM, NL cited in the application the whole document ---	
X	US 5 605 793 A (STEMMER WILLEM P C) 25 February 1997 (1997-02-25) cited in the application column 16, line 1 - line 6 column 16, line 50 - line 55 ---	47-60, 70-72, 74,75
X	WO 98 13487 A (MAXYGEN INC) 2 April 1998 (1998-04-02) page 39, line 30 - line 31; claims 1-57; figures 1,3 ---	58-60, 70-72,74

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHANG J-H ET AL: "Directed evolution of a fucosidase from galactosidase by DNA shuffling and screening" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 94, 1 April 1997 (1997-04-01), pages 4504-4509, XP002087464 ISSN: 0027-8424 the whole document ---	47-57,75
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A	CADWELL R AND JOYCE G ET AL: "Randomization of genes by PCR mutagenesis" PCR METHODS AND APPLICATIONS,US,COLD SPRING HARBOR, NY, vol. 2, 1992, pages 28-33, XP002087462 ISSN: 1054-9803 the whole document ---	47-57,75
A	FROMANT M ET AL: "DIRECT RANDOM MUTAGENESIS OF GENE-SIZED DNA FRAGMENTS USING POLYMERASE CHAIN REACTION" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 224, no. 1, 1995, pages 347-353, XP000486749 ISSN: 0003-2697 cited in the application the whole document -----	47-57,75

INTERNATIONAL SEARCH REPORT

Int :ional application No.
PCT/US 99/30925

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 76 (11-partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

In claim 11, SEQ ID Nos. 12 and 13 do not encode any amino acid sequence of claim 9 and 10.
Claim 76 refers to itself.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: (1-10,12-46,65-68,73)-complete, (11,58)-partially

A second luciferase that has enhanced resistance to an inhibitor of the luciferase relative to a first reference beetle luciferase; said second luciferase which comprises a plurality of amino acid substitutions relative to the reference beetle luciferase; said second luciferase wherein the reference luciferase is LucpPe-2 (luciferase from Photuris pennsylvanica); luciferases: luc 133-1B2 and luc146-1H2 which comprise SEQ ID Nos. 44 and 45; an isolated and purified nucleic acid molecule comprising a nucleic acid sequence selected from SEQ ID Nos.42 and 43; a vector comprising said nucleic acid molecule; a host cell comprising said vector; a fusion protein comprising said luciferase; the use of the second luciferase for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce a hybrid molecule, for high temperature reactions, or for creating luminescent solutions; a method for using said vector encoding said luciferase; a kit comprising: a container comprising said second luciferase; method to prepare an luciferase that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding luciferase which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding luciferase subjected to conditions that yield nucleotide mutations, wherein the luciferase encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the luciferase encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding luciferase that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the first polynucleotide sequence encodes Ppe2 and/or Ppl;

1.1. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc49-7C6, respectively SEQ ID Nos. 1 and 14, where the reference beetle luciferase is lucPpe-2;

1.2. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc78-0B10, respectively SEQ ID Nos. 6 and 19, where the reference beetle luciferase is lucPpe-2;

1.3. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc90-1B5 respectively SEQ ID Nos. 11 and 24, where the reference beetle luciferase is lucPpe-2;

1.4. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc81-6G01 respectively SEQ ID Nos. 13 and 26, where the reference beetle luciferase is lucPpl;

1.5. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc81-0B11, respectively SEQ ID Nos. 46 and 47, where the reference beetle luciferase is lucPpl;

1.6. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to lucPpe-2[T249M], respectively SEQ ID No. 12, where the reference beetle luciferase is lucPpe-2;

2. Claims: 47-57,75

A method to prepare an enzyme which is not a beetle luciferase and which has enhanced enzymological properties, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is not a luciferase and which has at least one enhanced enzymological property from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme which is not a luciferase, wherein the first isolated polynucleotide sequence is subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has at least one enhanced enzymological property relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences, wherein the selected isolated polynucleotide

sequence is subjected to oligonucleotide mediated mutagenesis with a plurality of oligonucleotides each comrising at least one codon that encodes a consensus amino acid which is not present in the first polynucleotide sequence; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme which is not a luciferase having at least one enhanced enzymological property and comprising a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the enzyme is DNA polymerase or RNA polymerase, chloramphenicol-acetyltransferase, beta-glucoronidase or beta-galactosidase; an enzyme which is encoded by the polynucleotide sequence of said method;

3. Claims: 58-partially, (59-64,69-72,74)-complete

A method to prepare an enzyme that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the further polynucleotide sequence encodes an enzyme that has increased thermostability relative to the first polynucleotide sequence, said method wherein said enzyme is DNA or RNA polymerase; a polynucleotide sequence obtained by said m

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 76 (11-partially)

In claim 11, SEQ ID Nos. 12 and 13 do not encode any amino acid sequence of claim 9 and 10.
Claim 76 refers to itself.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/30925

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